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ESTROGEN-INDUCED TUMORS OF THE KIDNEY IN THE SYRIAN HAMSTER

NATIONAL CANCER INSTITUTE MONOGRAPH NO. 1

DECEMBER 1959

THE NATIONAL CANCER INSTITUTE

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U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE

PUBLIC HEALTH SERVICE
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UNITED STATES OF AMERICA

Estrogen-Induced Tumors of the Kidney in the Syrian Hamster

by Hadley Kirkman

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE

PUBLIC HEALTH SERVICE • NATIONAL INSTITUTES OF HEALTH • NATIONAL CANCER INSTITUTE

DECEMBER 1959

National Cancer Institute Monograph No. 1

UNITED STATES GOVERNMENT PRINTING OFFICE, WASHINGTON : 1959

For sale by the Superintendent of Documents, U.S. Government Printing Office
Washington 25, D.C. - Price $1.25

Estrogen-Induced Tumors of the Kidney

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Estrogen-Induced Tumors of the Kidney. III. Growth Characteristics in the Syrian Hamster[1, 2, 3]

HADLEY KIRKMAN,[4] *Department of Anatomy, Stanford University School of Medicine, Stanford, California, and the Chester Beatty Research Institute, Institute of Cancer Research, Royal Cancer Hospital, London, England*

SUMMARY

The induction, latent period, growth rate, prevention, regression and viability, transplantation, and dependency *versus* autonomy of an estrogen-induced malignant, metastasizing renal neoplasm in the Syrian hamster are described and discussed. Investigation of these phenomena included: various hormones in addition to estrogens, *e.g.*, testosterone propionate, progesterone, deoxycorticosterone acetate, cortisone, and ACTH; and various surgical procedures, *e.g.*, gonadectomy, adrenalectomy, hypophysectomy, renal traumatization, and unilateral nephrectomy. The possible mechanism of estrogen tumorigenesis is discussed and the value of the hormonally induced renal carcinoma is emphasized, particularly in studies of carcinogenesis.

A KIDNEY tumor in an estrogen-treated Syrian hamster (*Mesocricetus auratus*, formerly *Cricetus auratus*) was first mentioned by Vasquez-Lopez (*1*), who regarded it as a metastasis from the estrogen-induced, pars intermedia, pituitary-gland tumor first described by him. There can be no serious doubt, however, that his "large, secondary deposit macroscopically visible in the left kidney" was a primary renal neoplasm such as that described originally by Matthews, Kirkman, and Bacon (*2*) and later, in more detail, by Kirkman and Bacon (*3–5*), Horning (*6–8*), Horning and Whittick (*9*), Kirkman (*10–12*), and others. At first these neoplasms were induced only in males and in gonadectomized hamsters of both sexes. Later (*10, 11, 13*) it was demonstrated that they also could be induced in

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[1] Received for publication December 29, 1958; revised May 25, 1959.

[2] This investigation was supported in part by research grants from the American Cancer Society, Inc., the National Cancer Institute, National Institutes of Health, Public Health Service, and the Jane Coffin Childs Memorial Fund for Medical Research.

[3] The experimental work reported here was completed at Stanford University; the data were assembled and the manuscript prepared during the author's tenure of a Senior National Science Foundation Fellowship (1957–58) and a Special U.S. Public Health Service Fellowship (1958–59) at the Chester Beatty Research Institute.

[4] I wish to thank the National Science Foundation, the Public Health Service, and the Chester Beatty Research Institute for making this study possible. My special thanks to Professor Eric Horning for numerous stimulating discussions and for reading the manuscript. The technical assistance of Drs. Masako A. Baba, Elizabeth M. Center, Mariano Obias, Robert Squire, and Sydney F. Thomas and of Fay Agate, Frank Barrett, Ray Crabbe, Gladys T. Kirkman, Max Millsap, Lee Nicholas, Yoshio Okumoto, and Marilyn Robbins, of Stanford University, and Michael J. Docherty, Robert McCullock, Kenneth Moreman, and Edward A. Sykes, of the Chester Beatty Research Institute, is gratefully acknowledged. Diethylstilbestrol and various hormones were generously supplied by the Ciba Pharmaceutical Products, Inc., Summit, N. J.

intact females, providing estrogen treatment was started during a period of relatively low progesterone secretion.

While it may be true that normal and neoplastic growth involves mechanisms older and more basic than hormonal ones, widespread use of hormonally induced tumors in investigations of carcinogenesis is highly desirable. The estrogen-induced renal tumor of the Syrian hamster is particularly well adapted to such studies in view of its ease of induction, its latent period of favorable duration, its sensitivity to inhibition by certain other hormones, and its prolonged period of progressive partial autonomy. With this in mind, the following detailed account of the growth characteristics of this tumor has been prepared.

A secondary tumor was considered metastatic, even though it is neither lymph nor blood borne. Since the most common sites of secondary renal tumors in the hamster are intra-abdominal, though many clinicians hesitate to refer to these as metastases, I follow the interpretations of Foulds (*14*) and Willis (*15*): "Tumour dissemination in the serous cavities from primary or secondary tumours of their viscera is a frequent and familiar event. Tumour particles are detached from the invasion sites and are carried by respiratory, peristaltic or other movements to remote parts of the cavity where they become implanted to form discrete metastatic nodules or plaques" (*15*).

MATERIALS AND METHODS

The hamsters were of heterogeneous origin: some purchased from dealers, some bred in our own animal quarters; none were of inbred stock. They were maintained in cages with wire bottoms—from 1 to 5 animals in a cage. Untreated, mature females were kept isolated. All were fed Purina laboratory chow *ad libitum*, with greens added twice a week. Room temperature was maintained at about 75° C.

For the most part, diethylstilbestrol (stilbestrol) and the various hormones were implanted subpannicularly as 20-mg. pellets[5] (30 mg. for testosterone propionate), and reimplanted every 150 days to insure constant absorption. The pellets were recovered at autopsy when possible, dried, and reweighed to calculate mean daily absorption. The pellets were placed between the shoulder blades through a small incision near the base of the tail; a pair of long, narrow forceps was used for this purpose.

Serial transfers, through the eighteenth subpannicular passage, were made by chopping up healthy bits of tumor tissue, with the aid of fine scissors, and injecting a few pieces subpannicularly through a long, 16-gauge hypodermic needle. Starting with the nineteenth transfer, this method was replaced by a more quantitative one. Healthy portions of the tumor were forced through a stainless steel screen, 30 meshes to the inch, by the rotary motion of a broad spatula. The screened tissue was then scraped off into a previously weighed vial containing 1 cc. of 0.9 percent NaCl solution. This was then diluted to a concentration that was usually 5 mg. of tumor per cc., and 1 cc. of this suspension was injected. With each method the needle was inserted near the base of the tail and the tumor tissue deposited slightly to the right of the dorsal midline at the level of the scapulae; light ether anesthesia was used. That the second method is superior to the first is indicated since a highly malignant, rapidly metastasizing, amelanotic melanoma, isolated on May 18, 1956, failed to grow at all in 2 females when transplanted by the early method, but showed uniformly excellent growth in 2 female hosts after transplantation of 1 and 5 mg. of screened tissue.

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[5] A satisfactory method of preparing pellets is described by McGregor, R. F., Putch, J. D., and Ward, D. N.: Estrogen-induced kidney tumors in the golden hamster. I. Biochemical composition during tumorigenesis. J. Nat. Cancer Inst. May, 1960.

RESULTS

Intrathoracic Transplantability

Metastases from primary renal tumors were transplanted, intrathoracically, into 6 orchiectomized hosts. The head of the male donor had been X-irradiated with 125 r weekly, for a total of 369 days (6625 r). The donor also carried stilbestrol pellets from the 69th through the 377th day of life. The hosts were killed 200 days after tumor transplantation. Only 3 showed transplant growth: 1 on the right parietal pleura, 1 in the anterior mediastinum and apex of the heart (fig. 1), and 1 subpannicularly at the injection site. A second group of 4 similar hosts received transplants from a male donor that had been implanted subpannicularly with a pellet of stilbestrol plus a pellet of stilbestrol : cholesterol (1:4) at 65 days of age and killed 465 days later; again metastases from primary tumors were used. When killed 214 days after tumor transplantation, none of the hosts showed transplant growth. Two animals in each group developed primary renal tumors. The mean average daily stilbestrol absorption for the 10 animals was 0.08 mg. The tumor did not grow well when transplanted intrathoracically.

Cheek-Pouch Transplantability

Cheek-pouch transplants were made only in gonadectomized animals; there is no reason why intact males would not have been equally satisfactory. These transplants did fairly well, but were not 100 percent successful, as shown in table 1.

Subpannicular Transplantability

To date, February 1959, the renal tumor has been carried through 44 successive subpannicular transfers. Thirteen different primary donors were used for the first transfer. With the exception of 5 orchiectomized, second-generation hosts that received transplants from a male donor,

Table 1.—Transplantability of renal tumor in hamster cheek pouch

Donor	Host sex	Number in sample	Mean age when stilbestrol implanted, with range	Mean duration of stilbestrol treatment, with range	Mean average stilbestrol absorption (*mg./day*)	Generation of serial transfer	Mean host age when given transplant, with range	Mean duration of transplant, with range	Number showing growth of transplant
1265* †	⚦	3	63 63–63	303 294–321	0. 07 0. 05–0. 09	1	175 175–175	191 182–209	2
1341‡ †	♀̸	6	50 49–50	179 134–200	0. 05 0. 03–0. 07	1	56 52–57	168 127–193	5
1335§	♀̸	4	49 49–50	153 150–155	0. 08 0. 05–0. 12	1	56 56–56	144 135–148	4
1460‖	⚦	4	79 79–80	370 342–410	0. 09 0. 08–0. 09	2	90 89–90	360 332–400	2
1267¶	♀̸	4	109 109–109	173 141–198	0. 09 0. 07–0. 13	2	116 116–116	166 134–191	3
1409**	⚦	3	40 40–40	164 164–164	0. 08 0. 05–0. 10	3	50 50–50	154 154–154	2
1409**	♀̸	1	42	141	0. 06	3	40	131	1
1407**	♀̸	6	31 31–31	212 143–246	0. 07 0. 04–0. 11	3	40 40–40	203 134–237	3

*Male; stilbestrol from 49th through 382d day of age; metastases used for transplant.

†The (⚦) sign, indicates orchiectomy; the (♀̸) sign, ovariectomy.

‡Male; stilbestrol from 69th through 446th day of age; 125 r X radiation to head, weekly, from 77th through 446th day of life (6625 r total); metastases used for transplant.

§Male; stilbestrol from 69th through 443th day of age; 125 r X radiation to head, weekly, from 77th through 443d day of life (6500 r total); metastases used for transplant.

‖Castrate; stilbestrol from 63d through 384th day of age; carried first serial transfer from 175th through 384th day of life; subpannicular transplant used for second serial transfer.

¶Male; stilbestrol from 50th through 352d day of age; carried first serial transfer from 150th through 352d day of life; subpannicular transplant used for second serial transfer.

**Spayed; stilbestrol from 109th through 274th day of age; carried second serial transfer from 116th through 274th day of life; subpannicular transplant used for third serial transfer.

all subsequent hosts, including 6 ovariectomized, second-generation ones, received transfers descended from an original primary renal tumor occurring in a male killed after 514 days of treatment with estradiol, implanted subpannicularly on the 50th day of life; the average daily absorption of the estrogen was 0.06 mg.

After the third transfer, with 5 unimportant exceptions, the transplant grew in 100 percent of the hosts. From the first to the 30th passage, 256 animals, the shortest latent periods for transplant palpability were 22 and 34 days. After the 30th transfer, latent periods of 15, 16, 17, and 18 days were observed. For the 88 animals dying spontaneously (table 2) the mean survival time, after transplantation, for the 34 series of transplant-bearing hosts was 118 days, with a range from 11 to 330 days. Although quite erratic, both the latent period for transplant palpability and the apparent survival time of the host evinced a tendency to become shorter with succeeding transfers; transplant growth, in mg. per day, tended to be greater with succeeding transfers, although this, too, was quite erratic. Actually the figures given for survival time in days were not significant, because the mean survival time of 118 days for the 88 animals dying spontaneously was exceeded by the mean survival time, 125 days, for the 182 animals whose death was induced. The range of the two sets of means emphasizes this lack of significance even more, being 11 to 330 days for the spontaneous group and 48 to 400 days for the group that was killed. Although the range between the two means could be reduced somewhat by eliminating the few animals that died before their transplants became palpable, on the assumption that death was due to some factor independent of the transplant, this was not considered statistically justifiable, since the same or some other irrelevant factor might have been acting on any of the other animals. The data are included to emphasize the importance of permitting *all* experimental animals to live as long as possible if *any* are to be used for calculating survival times. This importance is illustrated again among the untreated control groups in which, for females, males, and castrates, the maximum ages of animals that died spontaneously compared with those of animals killed were 600 to 790, 500 to 985, and 420 to 653 days, respectively.

For the entire series the mean age in days, when the stilbestrol pellet was implanted, was 109, with a range from 31 to 362; the mean duration of treatment was 135 days, with a range from 12 to 410; the mean absorption in mg. per day was 0.11, with a range from 0.28 for shorter periods to 0.03 for longer periods of treatment. The mean age when the transplant was made was 124 days, with a range from 31 to 369 days; the mean duration was 121 days, with a range from 11 to 400, including animals killed as well as those that died spontaneously; the mean average growth rate of the transplant was 100 mg. per day, with a range from 1 to 715 mg.; the maximum absolute weight attained by a transplant was 91 gm. as compared with a remaining carcass weight of 84 gm.; the average growth of this transplant was 929 mg. per day; the host was an intact, stilbestrol-treated female implanted with the 23d transfer at 180 days of

Table 2.—Incidence of metastases in stilbestrol-treated hosts carrying, through 34 serial passages, a renal tumor originally from an estradiol-treated, male donor

Number of hosts	Primary renal tumors		Mean average growth rate of transplant (*mg./day*)	Number of animals dying spontaneously	Mean maximum survival time after transplantation		Metastases				
	Left	Right			Spontaneous death	Induced death	Abdominal alone	Extra-abdominal alone	Abdominal plus extra-abdominal	None	Total
270	30	34	100	88	118	125	22 or 8%	11 or 4%	2 or 1%	235 or 87%	35 or 13%

life and killed 100 days later; the transplant was palpable after 1 month. The fact that the host was a female implies that a certain amount of autonomy may have been acquired by the tumor during the preceding transfers. Actually, the first indication of such partial autonomy was observed in the first serial transfer, as described later. A later paper in this series will present a discussion of the transition of the renal tumor from dependency to autonomy.

Of the 270 animals used as hosts, 34 developed primary tumors in the right kidney, and 30 of these 34 also developed tumors in the left kidney. Of the same total number of hosts, 35 showed metastases at autopsy; of these 35 animals, 22 did not develop primary tumors. The shortest observed latent period for metastases from a transplant was 59 days (21st serial passage).

From this series of 270 hosts, an additional 21 female hosts carrying transplants of primary renal-tumor metastases (4 hosts from one donor, 17 hosts from a second donor) were intentionally omitted. One of the group of 4, treated for 236 days, had a transplant weighing 99 mg.; the transplant from a second animal showed no growth after 325 days, but was histologically healthy. In this second series of 21 animals, 8 showed definite growth of the transplant. The earliest clear-cut evidence of growth was in a transplant weighing 26 mg. after 72 days in the stilbestrol-treated host. The largest transplant, 1394 mg., was found after 149 days of treatment (fig. 22). Expressed as average growth per day (9 mg.) this transplant compares favorably with the maximum growth rate of transplanted primary tumors in gonadectomized female hosts (8 mg.), but not so well with that in intact male hosts (21 mg.).

It is clear that the estrogen-induced renal tumor can be carried serially as an estrogen-dependent, subpannicular transfer in intact or gonadectomized hamsters of either sex. That even the first serial transfer will grow in the intact, mature female host is especially noteworthy since the tumor cannot be induced in such females, except during that portion of the estrous cycle characterized by the lowest level of progesterone secretion (*11*)—*i.e.*, between the middle of metestrum and the middle of proestrum, or at about 8 a.m. of day 1. It is clear, also, that the rate of transplant growth and the ultimate size attained by the transplant tend to increase with successive transfers. Although transplant growth is at first estrogen-dependent, metastases occur frequently and may involve intra-abdominal structures, the lungs (fig. 2), the heart (fig. 1), or the extra-abdominal lymph nodes (fig. 3).

Dependency

The dependency of the tumor is demonstrated by its failure to grow when transplanted subpannicularly in the untreated, intact, or gonadectomized host (table 3), and by the failure of the primary tumor to continue growth after estrogen is withdrawn (table 4). Not only does tumor growth stop after stilbestrol is removed but also the tumors undergo marked

Table 3.—Incidence of growth in renal tumors transplanted in untreated hosts*

Sex	Origin of transplant	Number of animals in sample	Average age when gonadectomized, with range (*days*)	Average duration of gonadectomy, with range (*days*)	Average age when transplanted, with range (*days*)	Average duration of transplant, with range (*days*)	Transplant growth
♀	Metastasis from primary tumor	7	—	—	32 32–32	200	None
⊗♀	Metastasis from primary tumor	10	47 47–47	196 130–203	49 49–49	194 128–201	None
⊗♂	Metastasis from primary tumor	10	41 41–41	212 212–212	49 49–49	204 204–204	None
♂	Metastasis from primary tumor	1	—	—	58	242	None
⊗♂	First serial transfer	10	53 53–53	253 253–253	152 152–152	200 200–200	None
⊗♂	Twelfth serial transfer	10	40 40–40	267 223–309	49 49–49	258 214–300	None
⊗♂	Thirteenth serial transfer	5	30 30–30	461 394–531	131 131–131	360 293–430	None
♂	Thirteenth serial transfer	13	—	—	109 98–117	237 180–301	1 of 13† (143 mg. or < 1 mg./day)
♀	Fourteenth serial transfer	17	—	—	161 128–177	195 6–350	None
♂	Fourteenth serial transfer	1	—	—	173	76	51 mg. or† < 1 mg./day
♀	Twentieth serial transfer	5	—	—	180 180–180	462 217–633	None

*All transplants were made subpannicularly, except for the first group of 7 females in whom transplants were made in the submaxillary glands.

†One of several autonomous variants of renal tumor, to be discussed in a later paper.

TABLE 4.—Failure of primary renal tumors to continue growth after removal of stilbestrol

Sex	Number of animals in sample	Mean age at autopsy, with range (*days*)	Mean age when stilbestrol pellet implanted, with range (*days*)	Mean age when gonadectomized, with range (*days*)	Mean duration of treatment with stilbestrol, with range (*days*)	Mean regression period, with range (*days*)	Healthy renal tumors at laparotomy before removal of stilbestrol	Indications of tumor regression, at autopsy	Histological regression
♂	3	428 422–459	50 50–50	—	253 253–253	134 119–156	3	3	3
⚦	17	593 416–605	51 50–51	51 50–51	250 250–250	292 115–305	No laparotomy performed	17	17
♀̸	1	419	52	52	367	117	1	1	1

regression, which is characterized first by a change from white to amber followed by a decrease in size, transformation into blisterlike vesicles filled with a clear, colorless fluid and, finally, by liquefaction. In spite of these marked indications of regression, the estrogen dependency applies to tumor growth rather than to tumor viability, as will be emphasized later.

Latent Periods

The latent periods for tumor induction and for transplant palpability are two different phenomena. In our series, the first 3 serial transfers averaged a latent period of about 100 days, as compared with a minimum of 163 days for tumor induction. Between the fourth and the twelfth transfers the average latent period decreased from about 100 to 50 days, and between the 13th and 30th transfers to about 25 days.

The effect of variations in estrogen dosage upon the latent period for the induction of primary lesions is somewhat uncertain. These are admittedly difficult data to collect, since there is no satisfactory way of determining at what point during treatment primary tumors first appear. Nevertheless, the observations recorded in table 5 do not disclose any clear-cut differences after the administration of one or five 20-mg. pellets of stilbestrol in ovariectomized hamsters. Among the 15 spayed animals receiving a single pellet, none survived for the 250-day period of treatment, after which we ordinarily expect to find grossly visible renal tumors in approximately 100 percent of animals (*5*). Even so, 6 of the 15 developed renal tumors; the shortest latent period was 163 days. Among the 9 animals receiving 5 times the above dose of stilbestrol, 2 survived for 250 days or longer, but only 1 of the 2 had recognizable tumors at autopsy; 3 others, however, did have such lesions after 131, 184, and 200 days. Although this difference is in favor of the group receiving the heavier dosage, it is very slight in comparison with the difference in dosage. Overall, there is little if any significant difference between the results after the 2 dosages.

Nevertheless it cannot be concluded that the potency of the estrogenic stimulus has no significant effect on the latent period of the induced tumor. Certain estrogens (table 6) require much longer periods of treatment than that required by either estradiol or stilbestrol for renal-tumor induction. Omitting the castrates from the table, the mean latent period for the remaining 4 groups was estimated at 428 days, compared with 250 days for stilbestrol- or estradiol-treated males. In earlier reports (*5, 16*) tumors had not yet been induced either by ethynyl estradiol or doisynolic acid, and estrone and estriol had not yet been used.

The combination of stilbestrol with cholesterol, however, does appear to affect the latent period for tumor induction (table 5). It is not surprising (*5*) that a single subpannicularly implanted 20-mg. pellet of stilbestrol:cholesterol, in a 1:4 ratio, failed to result in renal tumors, for such a pellet contains only 4 mg. of the estrogen as compared with the 20 mg. ordinarily used. By administering 5 of these mixed pellets simultaneously, results comparable to those achieved with a single 20-mg. pellet of pure stilbestrol were anticipated. However, 5 of the 8 males failed to acquire any renal tumors. One had a microscopic tumor in 1 kidney, 2 had macroscopic tumor nodules in each kidney, and in 1 of these 2 animals the tumors (fig. 20) had metastasized (fig. 21). The figures concerning total and average absorption of the pellets make it clear that tumor incidence and duration of latent period in the different groups are markedly affected by cholesterol when combined with stilbestrol. The mean average absorption rate for pure estrogen in the 11 males developing bilateral tumors was 0.08 mg. per day, as contrasted with 0.02 mg. per day for the 2 males receiving 5 of the mixed pellets and developing bilateral tumors. This is a lower dosage than the 0.03 mg. of stilbestrol in 1 cc. of sesame-oil solution, injected subpannicularly, and earlier found ineffective in inducing renal tumors (*5*). This suggests that there may be significant factors involved in dosage which are separate from absorption rates. That the effect of cholesterol may be confined to interference with stilbestrol absorption is indicated by the occurrence of large tumors in each kidney of 9 of 10 males (6 of them with metastases) receiving pure stilbestrol in addition to stilbestrol and cholesterol in combination.

That functional stimulation of the kidney through unilateral nephrectomy might shorten the latent period for tumor induction was suggested by Horning (*6*). Following up this suggestion we obtained the results recorded in table 7. Since these results do not agree with those of Horning, both observations will be discussed later.

Prevention of Tumor Induction

With few exceptions, the induction of renal tumors by stilbestrol can be prevented by implanting pellets of testosterone propionate, deoxycorticosterone acetate (DCA), or progesterone, with the stilbestrol (table 8). The only failures were: (1) A male treated with stilbestrol and testosterone propionate from the 116th day of life, for 359 days, with a total absorption of 45 mg. of the estrogen and 70 mg. of the androgen, and an average daily absorption of 0.13 mg. of the former and 0.20 mg. of the latter. At autopsy tiny tumors were visible in each kidney; these were verified by histological examination (fig. 5). (2) A male similarly treated from the 50th day, for 434 days, with an average daily absorption of 0.14 mg. of stilbestrol and 0.20 mg. of testosterone propionate. A microscopic tumor was found in the left kidney. (3) A male treated with stilbestrol and DCA from the 64th day of life, for 289 days, with a total absorption of 25 mg. of the estrogen and 31 mg. of the DCA, and an average daily absorption of 0.09 mg. of the former and 0.11 mg. of the latter. At autopsy no tumors were observed in either kidney, but subsequent histological examination of the right kidney disclosed a microscopic tumor nodule. None of the 3 inhibitors had any tumorigenic action on the kidneys, in the dosages used (table 9).

Table 5.—Latent period for induction of the estrogen-dependent renal tumor

Sex	Treatment	Tumors	Number of animals in sample	Average age at start of treatment, with range	Average duration of treatment, with range	Average stilbestrol absorption (*mg.*)	Mean average stilbestrol absorption (*mg./day*)	Average stilbestrol : cholesterol absorption (*mg.*)	Mean average stilbestrol : cholesterol absorption (*mg./day*)	Average tumor size
♀	One 20-mg. pellet of stilbestrol, subpannicular	None	9	77 30–372	159 98–241	15 6–25	0.09 0.06–0.10	—	—	—
		Unilateral	1	95	222	26	0.11	—	—	Microscopic
		Bilateral	5	47 30–52	205 163–246	16 9–21	0.07 0.03–0.10	— —	—	Microscopic to small
		Metastases	0	—	—	—	—	—	—	—
♀	Five 20-mg. pellets of stilbestrol, subpannicular	None	5	50 49–51	161 42–257	57 21–94	0.38 0.20–0.50	—	—	—
		Unilateral	2	51 50–51	158 131–184	74	0.40	—	—	Medium
		Bilateral	2	51 51–51	239 200–277	108 81–135	0.44 0.40–0.48	— —	—	Medium to large
		Metastases	1	50	277	135	0.48	—	—	Large
♂	One 20-mg. pellet of stilbestrol, subpannicular	None	14	58 49–68	169 90–330	16 7–30	0.10 0.05–0.13	—	—	—
		Unilateral	3	62 50–68	213 204–217	17 16–18	0.08 0.08–0.09	—	—	Microscopic
		Bilateral	11	55 50–68	269 220–305	22 12–33	0.08 0.05–0.11	—	—	Large
		Metastases	4	54 50–67	273 229–304	26 21–29	0.10 0.09–0.10	—	—	Large
♂	One 20-mg. pellet of stilbestrol plus 1 of stilbestrol : cholesterol (1 : 4)	None	1	57	328	31	0.09	2	0.01	—
		Unilateral	0	—	—	—	—	—	—	—
		Bilateral	9	63 57–65	410 272–522	38 25–51	0.09 0.08–0.10	2 0–6	0.00 0.00–0.02	Large
		Metastases	6	64 57–65	413 345–521	39 33–48	0.10 0.09–0.10	3 0–6	0.01 0.00–0.02	Large

♂One 20-mg. pellet of stilbestrol : cholesterol (1 : 4), subpannicular	None	22	53	216	—	—	0	0. 00	—
			50–63	22–502			0–1	0. 00–0. 01	
	Unilateral	0	—	—	—	—	—	—	—
	Bilateral	0	—	—	—	—	—	—	—
	Metastases	0	—	—	—	—	—	—	—
♂Five 20-mg. pellets of stilbestrol : cholesterol (1 : 4), subpannicular	None	5	50	370	—	—	0	0. 00	—
			49–50	296–406			0–0	0. 00–0. 00	
	Unilateral	1	50	406	—	—	0	0. 00	Microscopic
	Bilateral	2	49	424	—	—	7	0. 02	Medium to large
			49–49	424–424			0–13	0. 00–0. 03	
	Metastases	1	49	424	—	—	13	0. 03	Large

Table 6.—Latent periods for renal-tumor induction by several estrogens

Sex	Estrogen	Mean age when treatment started, with range	Mean duration of treatment, with range	Number of animals in sample	Number with renal tumors in 1 or both kidneys	Estimated mean latent period, with range
♂	Estriol	49 48–50	513 318–601	11	6	514 396–593
♂	Estrone	50 49–51	465 372–602	8	7	410 350–550
♂̸	Estrone	50 50–50	409 359–416	10	9	389 300–400
♂	Ethynyl estradiol	49 49–50	388 301–400	17	5	388 350–400
♂	Fenocylin*	51 48–76	397 301–467	12	1	400

*7-Methylbisdehydrodoisynolic acid.

The efficacy of progesterone in preventing tumor induction is consistent with the general failure of estrogen to induce renal tumors in intact females and with its success in inducing them in ovariectomized animals and in other females characterized by low levels of progesterone secretion (*11*).

Regression and Viability

Attempts to cause tumor regression have involved the use of transplants, primary tumors, and metastases from each. As indicated in table 4, the withdrawal of estrogen for 115 days, or longer, from primary renal-tumor-bearing hamsters, whether intact males or gonadectomized of either sex, is followed by gross and histological indications of tumor regression (figs. 4 and 6). If, after these indications of regression have been established, stilbestrol is again implanted, the apparently regressed tumor nodules within 57 days or less (table 10) regain their healthy, white appearance and resume growth. This occurs even after a regression period of 200 days.

A second attempt to bring about the regression of primary tumors consisted of subpannicular implantation of 30-mg. pellets of testosterone propionate, after verifying the existence of tumors at laparotomy (table 11). No regression occurred in the 15 males, with one exception. This animal had small renal tumors after 303 days of stilbestrol treatment, and then testosterone propionate was added to the stilbestrol for an additional 137 days before it died. At autopsy no tumors were found on the surface or in the interior of either kidney; neither gland was sectioned. In this experiment none of the animals showed apparent metastases at laparotomy, but at autopsy metastases were found in 5; in 3 of these, they were so abundant as to demonstrate an unmistakable increase after the implantation of androgen (figs. 7 and 8).

A certain amount of regression occurred in transplants of the sixth and tenth generations after the removal of stilbestrol in orchiectomized hosts (table 12). In all instances the transplants were easily palpable

Table 7.—Effect of unilateral nephrectomy on the induction of renal tumors in male hamsters implanted subpannicularly with 20-mg. pellets of stilbestrol

Treatment	Average age when left kidney removed, with range	Average age when stilbestrol implanted, with range	Average duration of treatment, with range	Stilbestrol absorption, with range (*mg./day*)	Renal tumors at autopsy		Average size of renal tumors		Metastases	Number of animals in sample	Remarks
					Left	Right	Left	Right			
Nephrectomized	54 51–77	54 51–77	231 231–231	0. 11 0. 09–0. 13	—	7	—	Small	0	8	At laparotomy: no tumors after 150 days' treatment. At autopsy: minute tumors in 2 of 8 after 200 days; no tumors in 1 of 8 after 231 days; minute tumors in 4 of 8 after 231 days; medium tumors in 2 of 8 after 231 days; large tumors in 1 of 8 after 231 days.
Intact	—	60 49–77	228 204–260	0. 10 0. 05–0. 14	5	7	Medium	Small	1	12	At autopsy: no right kidney tumors in 5 of 12 after 228 days; microscopic right kidney tumors in 3 of 12 after 228 days; small right kidney tumors in 3 of 12 after 228 days—includes 1 animal with huge left kidney tumor and abundant metastases—medium right kidney tumors in 1 of 12 after 228 days.

TABLE 8.—Inhibition of renal-tumor induction by combining stilbestrol with testosterone and/or testosterone propionate, deoxycorticosterone acetate, or progesterone pellets implanted subpannicularly

Sex	Mean age when gonadectomized, with range (*days*)	Mean age when treatment with inhibitor plus stilbestrol started, with range (*days*)	Mean duration of treatment, with range (*days*)	Inhibitor used	Mean age when autopsied, with range (*days*)	Number of animals showing renal tumors		Number of animals in sample	Manner of administration of inhibitor	Manner of administration of stilbestrol
						Left	Right			
♂	—	50 50–50	452 271–543	Testosterone	502 321–593	0	0	10	Injected plus pellet*	Injected plus pellet†
♂	—	59 40–181	358 228–543	Testosterone propionate	418 287–604	2	1‡	56	30-mg. pellet implant. subpan.	20-mg. pellet implant. subpan.
⚦	36 28–43	50 50–51	408 392–458	Testosterone propionate	480 442–518	0	0	17	"	"
♀	—	79 41–181	310 223–406	Testosterone propionate	383 273–509	0	0	57	"	"
♀̸	42 40–44	50 50–51	307 261–400	Testosterone propionate	357 311–451	0	0	8	"	"
♂	—	60 55–64	315 219–380	DCA	384 283–444	0	1§	11	20-mg. pellet implant. subpan.	"
♂	—	50 50–50	361 293–452	Progesterone	397 343–482	0	0	11	"	"
⚦	55 50–63	55 50–63	399 399–400	Progesterone	454 450–462	0	0	14	"	"
♀	—	51 50–51	329 243–399	Progesterone	380 294–450	0	0	15	"	"
♀̸	58 57–59	58 57–59	287 219–401	Progesterone	345 278–460	0	0	13	"	"
♂	—	51 51–51	457 336–503	Progesterone plus testosterone propionate	508 387–554	0	0	7	20-and 30-mg. pellets implant. subpan.	"
⚦	44 40–46	53 49–59	419 322–482	"	472 373–533	0	0	13	"	"
♀	—	51 51–51	235 214–287	"	285 265–338	0	0	5	"	"
♀̸	45 42–47	51 51–51	300 239–400	"	351 290–451	0	0	4	"	"

*Subpannicular injection of 1.0 mg. in 2 cc. saline suspension every 2 days; followed by implantation of 30-mg. pellet.

†Subpannicular injection of 0.6 mg. in 2 cc. saline suspension every 2 days; followed by implantation of 20-mg. pellet.

‡A microscopic tumor nodule was found in the left kidney of 1 animal treated for 434 days, and in each kidney of 1 animal treated for 359 days.

§A microscopic tumor nodule was found in 1 animal treated for 289 days.

Table 9.—Absence of renal tumors in hamsters treated with testosterone and/or testosterone propionate, deoxycorticosterone acetate, or progesterone

Sex	Mean age when gonadectomized, with range (*days*)	Mean age when hormone was implanted, with range (*days*)	Hormone used	Mean age when autopsied, with range (*days*)	Number of animals in sample	Number of animals showing renal tumors at autopsy		Mean duration of treatment, with range
						Left	Right	
♂	—	87	Testosterone*	492	1	0	0	405
♀	—	87	Testosterone*	413	1	0	0	326
♂	—	64 50–320	Testosterone propionate†	678 276–958	20	0	0	614 276–908
♂̸	48 46–48	50 50–50	Testosterone propionate†	831 666–956	15	0	0	781 616–906
♀	—	50 50–50	Testosterone propionate†	538 450–743	12	0	0	488 400–693
♀̸	46 45–48	50 50–50	Testosterone propionate†	697 360–798	14	0	0	647 310–748
♂	—	50 50–50	DCA, 20-mg. pellet implant. subpan.	369 300–449	8	0	0	319 250–399
♂	—	50 50–50	Progesterone, 20-mg. pellet subpan.	465 463–468	15	0	0	415 413–418
♂̸	47 46–50	50 50–50	Progesterone, 20-mg. pellet subpan.	460 420–464	15	0	0	410 370–414
♀	—	50 50–50	Progesterone, 20-mg. pellet subpan.	442 288–464	15	0	0	392 238–414
♀̸	48 47–48	50 50–50	Progesterone, 20-mg. pellet subpan.	454 318–465	14	0	0	404 268–415

*Subpannicular injection of 1.0 mg. in 2 cc. saline suspension every 2 days.

†Subpannicular implantation of 30-mg. pellet every 150 days.

Table 10.—Growth of primary, estrogen-induced, renal tumors after regression periods of 100 and 200 days

Accession No. of host	Sex	Age at autopsy (*days*)	Age when implanted with 20-mg. pellet of stilbestrol	Duration of stilbestrol treatment	Duration of regression period	Age when stilbestrol reimplanted	Duration of 2d period of treatment	Condition of renal tumors after:						
								252 days of stilbestrol	352 days of stilbestrol	60 days of regression	100 days of regression	200 days of regression	157 days of stilbestrol renewal	103 days of stilbestrol renewal
2554	♂	559	50	252	200	502	57	Small, healthy	—	Amber-colored	—	Clear blisters	Small, healthy	—
2600	♂	605	50	252	200	502	103	Small, healthy	—	Amber-colored	—	Clear blisters	—	Small healthy
2599	♂	605	50	352	100	502	103	None seen	Small, healthy	—	Clear blisters	—	—	Small, healthy

Table 11.—Growth of primary, estrogen-induced, and estrogen-dependent renal tumors in male hamsters, after addition of testosterone propionate to the stilbestrol

Number of animals in sample	Average age at autopsy, with range (*days*)	Average age when stilbestrol pellet implanted, with range (*days*)	Average age when testosterone propionate pellet implanted, with range (*days*)	Average duration of stilbestrol treatment, with range	Average duration of testosterone propionate treatment, with range	Average age when renal tumors seen at laparotomy, with range	Number of animals showing renal tumors at autopsy		Number of animals showing metastases at autopsy	Number of animals showing evidence of renal-tumor regression at autopsy
							Left	Right		
15	498 352–557	50 50–52	310 303–353	448 302–507	188 48–254	310 303–353	13	14	5	1

before removal of the pellets. In 3 of 20 hosts no transplant was found at autopsy. Marked regression was observed in the others, but only 3 of these had any histologically healthy tumor tissue (fig. 9). The maximum regression period was 281 days. In sharp contrast with these 11 castrates was a single intact male host, #2414. In this animal the stilbestrol was not removed until the transplant was quite large (6 × 3 × 3.5 cm.), but this was true also of 4 of the castrates. During the 1st month of the ensuing regression period, the transplant diminished slightly in size (5.5 × 2.5 × 2 cm.). At autopsy, 122 days after stilbestrol was removed, the transplant still measured 3.5 × 3 × 3 cm. and weighed almost 13 gm. It consisted for the most part of necrotic tissue, but healthy growing areas were present at the periphery of the mass. Two small metastases occurred in the lungs; these showed no sign of regression (fig. 10). The testes and accessories of this animal were still moderately atrophic, the left testis weighing 583 mg. as opposed to about 1700 mg. for a left testis from a normal, untreated male of the same age. A second intact host, #3491, with a regression period of only 21 days, had a small, highly necrotic transplant at autopsy, although the transplant was a large one when the stilbestrol was removed. The left testis weighed 1114 mg. as compared with about 1600 mg. for a left testis of a normal, untreated male of the same age.

TABLE 12.—Regression in serial transfers of the estrogen-induced renal tumor in orchiectomized and intact male hosts after cessation of stilbestrol treatment*†

Size of sample	Serial transfer	Mean age at autopsy, with range (*days*)	Mean duration of treatment, with range	Mean duration of regression period, with range	Mean age when transplanted, with range	Mean size of transplant, with range (*mg.*)
11 ⚦	6	218	114‡	63	58	Medium§
		137–290	66–160	30–90	57–60	0‖–731
9 ⚦	10	378	89¶	244	45	Minute**††
		311–392	65–126	200–281	45–46	5–12
♂#3491	11	285‡‡	152	21	112	201
♂#2414	10	608§§	175	122	318	12830

*All orchiectomies were performed on the 40th day of life.

†None of the transplants in these animals metastasized.

‡Stilbestrol implanted on the 40th day of life.

§Gross indications of regression visible at autopsy in all. None were transplanted into new hosts.

‖No transplants found at autopsy in three.

¶Stilbestrol implanted on the 45th day of life.

**Gross indications of regression visible at autopsy in all. Five were transplanted into new stilbestrol-treated hosts; growth occurred in all.

††Transplant remnants found at autopsy in all.

‡‡Stilbestrol implanted on the 112th day of life.

§§Stilbestrol implanted on the 311th day of life.

It is difficult to account for the lack of regression in the one intact male, #2414, except in terms of a transition from dependency to autonomy on the part of some of the cells in the transplant. While it is true, as shown in table 13, that the transplant, once well established under the influence of stilbestrol, can be maintained in an uninterrupted state of

TABLE 13.—Failure of testosterone propionate, progesterone, and deoxycorticosterone acetate to destroy viability of seventh serial transfer of estrogen-induced renal tumor, when given with stilbestrol*

Antagonist used	Sex	Number of animals in sample	Average age at autopsy, with range	Average duration of stilbestrol treatment, with range	Average age when transplant became palpable, with range†	Average age when antagonist was given, with range	Average duration of treatment with antagonist, with range	Average weight of transplant at autopsy, with range (*mg.*)	Average growth rate of transplant, with range (*mg./day*)	Indications of transplant regression
Testosterone propionate	♂	9	215 143–227	184 112–196	86 51–132	101 82–132	114 36–145	52769 6132–122000	287 31–575	None
Progesterone	♂	7	211 148–226	180 117–195	99 82–128	99 82–128	112 41–144	2827 552–8104	16 3–42	None
DCA	♂	9	170 120–227	139 89–196	101 82–132	101 82–132	68 13–145	9457 88–55000	68 7–2806	None

*Stilbestrol implantations and tumor transplantations were performed on the 31st day of life.

†In 2 cases the transplant was not palpable when testosterone propionate was implanted, *i.e.*, androgen given on the 82d day of life and transplant palpable on the 106th day of life. In all other instances the transplant was readily palpable at the time the antagonist was administered.

growth by testosterone propionate alone, it cannot be assumed that, in the case of #2414, the testes were secreting enough androgen to account for the healthy transplant and for pulmonary metastases. If this were true, the transplant carried by #3491 should not have undergone necrosis.

That transplants of the sixth, tenth, and eleventh serial transfers (table 12) contained variable numbers of autonomous tumor cells, in addition to many estrogen-dependent ones, is suggested by the fact that in four instances, where the necrotic remnant of a transplant was retransplanted to a new, estrogen-treated, intact, male host, growth of the transplant was clearly evident, *e.g.*, 4000 mg. after 94 days, and 6693 mg. after 143 days in three and probable in the fourth. In a fifth instance of retransplantation, the donor transplant consisted of a tiny beaded string of translucent nodules, in which necrosis was doubtful; this string may have consisted entirely of autonomous cells. The question of autonomy will be considered in detail in a later paper.

Attempts to destroy transplant viability by administering either testosterone propionate (fig. 11), progesterone, or deoxycorticosterone acetate were unsuccessful (table 13). Of the 9 animals receiving the androgen, the average growth of the transplant over the total period of treatment was 287 mg. per day; with DCA this was reduced to 68 mg. and with progesterone to 16 mg. In 4 stilbestrol-treated, male controls carrying the seventh serial transfer for 106 to 191 (mean 156) days, the average growth rate of the transplant varied from 62 to 230 (mean 126) mg. per day, which suggests that with testosterone propionate the growth rate is increased and that with DCA and progesterone the growth rate is decreased. In all 3 groups mitotic figures were evident in the transplants; no comparative counts of dividing cells have been made as yet.

With this possible effect of testosterone propionate in mind, a series of 10 orchiectomized hamsters was given the eleventh serial transfer of the tumor and stilbestrol at the same time. When the transplants became palpable, the stilbestrol was removed and testosterone propionate implanted in its place. The animals were autopsied at a mean age of 313 days, after a mean length of stilbestrol and androgen treatment of 68 and 197 days, respectively. The mean transplant weight was 73.5 gm., representing a mean average growth rate of 307 mg. per day.

To check this apparent stimulating action of testosterone propionate on the growth of the transplant, tumor tissue from 3 of the animals was retransplanted, with stilbestrol, into 6 intact, male hosts. After a mean treatment period of 125 days, the animals were autopsied—the mean transplant weight was 20.5 gm., representing a mean average growth rate of 164 mg. per day.

It seems reasonable, therefore, that the eleventh and twelfth serial transfers of the tumor have a faster growth rate with testosterone propionate than with stilbestrol treatment. This does not justify the assumption, however, that testosterone propionate is at least as effective as stilbestrol in establishing the newly transplanted tumor, for when tumor tissue from the same 3 donors used previously was transplanted into 6 males given testosterone propionate alone, at the same time, the mean average growth rate, after a mean duration of 141 days, was only 71 mg. per day, with a mean transplant weight of 10 gm. The transplants were healthy, however, and contained many mitotically dividing cells (fig. 12).

That a certain degree of autonomy had been attained by this time is indicated further by the fact that when portions of the same samples were transplanted into 6 intact, untreated males, the tumor grew in 2 of them and attained weights of 242 and 752 mg., respectively, in 153 days; for these 2 animals the mean average growth rate was 3 mg. per day.

To test indirectly for autonomous cells in these transplants, primary renal-tumor tissue was transplanted subpannicularly in ten 183-day-old males, implanted with 30-mg. pellets of testosterone propionate at the same time. From 224 to 298 (mean 275) days later, transplant remnants were recovered at autopsy from only 2 of the hosts; no growth was evident in either. Testosterone propionate, therefore, cannot be substituted for estrogen in bringing about the establishment and subsequent growth of the first serial passage of the renal tumor.

With DCA also, in place of testosterone propionate, there was growth in each of 2 intact male hosts. After 74 and 153 days the transplants weighed 40 and 528 mg., showing average growth rates of <1 and >3 mg. per day.

That the maintenance of transplant growth by testosterone propionate alone is not due to a promoting action by the androgen after initiation by stilbestrol is indicated by the failure of the androgen to support growth of the first serial passage.

The fact that growth of the seventh serial transfer in stilbestrol-treated males was not entirely stopped by the addition of progesterone (table 13) suggested that the transplant might be induced to grow in stilbestrol-treated female hosts. To test this possibility, metastases from primary renal neoplasms of a male donor treated with stilbestrol for 480 days, plus testosterone propionate during the final 227 days, were transplanted subpannicularly in 4 intact female hosts that received 20-mg. pellets of stilbestrol subpannicularly at the same time. In 3 of the hosts, treated for 162, 310, and 325 days, no transplant growth occurred, but in the 3 treated longest, the transplant was recognizable and was histologically healthy. In the remaining animal treated for 236 days, the transplant (fig. 13) weighed 99 mg., representing an average growth rate of <1 mg. per day, as compared with a mean rate of 16 mg. per day for the seventh transfer in stilbestrol : progesterone-treated males.

Presumably this growth rate in female hosts would have been greater had the transplant represented the seventh instead of the first serial transfer. This transfer was not made, but transfers from the 15th, 16th, 17th, 21st, 22d, 23d, and 26th series were made (table 14), with transplant growth occurring in all; the average transplant weight, over an average of 101 days, exceeded 19 gm., with a mean average growth rate of 192 mg. per day. The significance of this finding with reference to autonomy is to be considered in a later paper.

Table 14.—Growth of serial transfers of the estrogen-induced renal tumor in stilbestrol-treated female hosts

Serial transfer	Number of animals in sample	Average age at autopsy, with range	Average age at start of treatment with stilbestrol and transplant, with range	Average duration of treatment, with range	Average weight of transplant, with range (*mg.*)	Average growth rate of transplant, with range (*mg./day*)	Metastases	Average age when transplant became palpable, with range	Number of animals showing transplant growth
1	23	249	126	123	8	1	—	146	14
		175–466	123–141	52–325	0–1394	0–9		91–236	
16	3	210	62	148	2821	19	—	123	3
		210–210	62–62	148–148	351–6055	2–41	—	123–123	
17	10	218	162	56	676	12	—	51	10
		213–218	162–162	51–56	21–2265	<1–40		51–51	
18	1	190	74	116	497	4	—	116	1
22	1	111	48	63	24000	381	Pulmonary	31	1
23	2	279	180	99	70000	710	—	39	2
		278–280	180–180	98–100	49000–91000	490–929	—	39–39	
24	1	234	79	155	18000	116	Cardiac	44	1
27	1	325	255	70	20000	286	—	31	1
Mean	42	224	123	101	19351	192	2 or	62	33 or
		111–325	48–255	56–155	497–70000	4–710	5%	31–123	79%

Cortisone appears to increase the incidence of primary renal tumors and of metastases (figs. 14 and 15) in stilbestrol-treated, transplant-bearing male hosts, as indicated in table 15. In view of the 25-day difference in the mean duration of treatment, the 35 mg. per day difference in the mean average rates of transplant growth may not be significant, but the difference in the incidence of primary tumors and of metastases is significant. Unpublished data indicate that cortisone alone will not maintain transplant growth in the castrated male or female or in the adrenalectomized, spayed hamster (fig. 16), although some healthy tumor cells (fig. 17) may be found in otherwise markedly regressed (fig. 18) transplants 100 days after the removal of stilbestrol and the administration of cortisone. The transplant remains healthy and growing in the cortisone-maintained, adrenalectomized castrate, providing stilbestrol treatment is continued (fig. 19).

DISCUSSION

Various laboratories have reported difficulty, and even complete failure, in attempts to induce renal tumors in hamsters by treatment with stilbestrol. Both Horning (*17*) and I have occasionally had refractory hamsters, *e.g.*, my #718, which was treated and autopsied by graduate students, showed no renal tumors after 444 days of treatment. It is possible that certain inbred strains may not develop the tumors; it is interesting, however, that two laboratories of the same institute, using the same strain of hamster and stilbestrol pellets from the same source, have had contradictory results—one reporting 90 to 100 percent success, the other no success. In general, it is suspected that most failures are due either to the substitution of "tablets" for pellets (tablets are commercially available for oral administration and are quickly absorbed even when implanted subcutaneously; coating them with wax slows down absorption, but does not result in a high incidence of tumors; satisfactory pellets are composed of pure estrogen compressed under high pressure) or to the dilution of pellets with cholesterol or other binding medium. It is possible that some failures have been due to other factors; *e.g.*, failure to maintain a constant supply of estrogen; failure to use adequate doses of pure stilbestrol or estradiol; failure to allow for the long latent period of induction; the use of females; or possible nutritional differences, though this is considered unlikely. In our work we have used a highly heterogeneous stock, including some partial albinos, some blind albinos, and hybrids between partial albinos and golden hamsters. All have yielded high incidences of renal tumors, usually 100 percent. Horning has had similar success with "champagne" hamsters (*17*).

That this dependent, malignant, renal tumor is transplantable in estrogen-treated, ovariectomized hosts was first announced in 1951 (*18*). The following year metastases from 200-day-old subpannicular transplants in 7 stilbestrol-treated, orchiectomized hosts were reported (*4*). In addition to one metastasis in a cervical lymph node, all 7 animals had

TABLE 15.—Effect of cortisone upon incidence of primary renal tumors and of metastases in stilbestrol-treated, transplant-bearing male hamsters

Treatment	Serial transfer used	Number of animals in sample	Mean age treatment started, with range	Mean duration of treatment, with range	Mean weight of transplant, with range (*gm.*)	Mean average growth of transplant, with range (*mg./day*)	Number of animals with primary renal tumors	Number of animals with metastases
Stilbestrol	11th to 13th, inclusive	10	176 54–207	145 67–207	37 2–84	246 17–564	1	2
Stilbestrol plus cortisone	11th	8	51 51–51	170 162–179	48 12–81	281 51–453	7	6

metastases within the abdominal cavity, though no primary renal tumors were found. The transplantability of the neoplasm in estrogen-treated male hosts was described in great detail by Horning (*6–8*). Since all Horning's hosts had primary renal tumors and growing transplants, he suspected (*8*) that growth of the transplant might be dependent on the presence of the primary lesions, as well as on exogenous estrogen, and cited certain other tumors in which a comparable dependency had been reported. Since, even in the first serial transfer, quite a number of the stilbestrol-treated hamsters reported here (table 2) had shown actively growing transplants, and even metastases, prior to the appearance of primary renal lesions, it is apparent that no such dependency exists and that the latent period for transplant palpability is often very significantly shorter than the latent period for primary tumor induction.

That the renal tumor will not grow after transplantation in hosts not treated with exogenous estrogen has been fully demonstrated (*6, 19–21*).[6]

The mechanism by which estrogen becomes carcinogenic for the hamster kidney has been discussed briefly (*6*), but remains unknown. It has been suggested by Horning (*6*) that the hamster liver may not be endowed with as high an estrogen-inactivating power as livers of other rodents are, and that this might be one of the reasons why estrogens induce renal tumors in this species alone. However, the hamster liver appears to be quite effective in the inactivation of estradiol, since we find that renal tumors fail to develop in intact and orchiectomized males after the implantation of estradiol pellets in the spleen for as long as 300 days (unpublished data). Contrary to our earlier suggestion (*5*) this indicates that the estrogen itself, rather than one of its metabolites, is responsible for tumor induction.

It is quite possible that the hamster is not unique in its capacity to develop renal tumors under prolonged estrogen treatment. Most rodents, so treated, die at a relatively early age from effects of the treatment, *e.g.*, mammary- or pituitary-gland tumors; longer survival might be associated with the appearance of kidney tumors. This possibility is recognized by Richardson (*22*) who found renal adenocarcinomas in 2 of 100 male mice that did not develop mammary-gland tumors under stilbestrol treatment. Also, as discussed in (*12*), there are theoretical reasons for suspecting that it may be possible to induce tumors in the reptilian metanephros, by means of estrogen treatment.

The hamster, like the rat and mouse, develops huge pituitary-gland adenomas under prolonged estrogen treatment (*1, 23*). These glands include enlarged anterior lobes rich in acidophilic cells similar to those which, in the rat, are interpreted as being prolactin-secreting (*24*). Unlike the rat and the C3H mouse (*25*), however, the estrogen-treated hamster acquires no mammary-gland tumors; in fact no stimulation of any part of the mammary gland has been observed at autopsy in any of my hamsters under any of the experimental conditions described in this paper. Neither has Horning (*17*) observed any stimulation of these glands in estrogen-treated hamsters. In more than 30 different types of neoplasms observed in my colony no mammary-gland tumors are included. I have found only two reports of such tumors in the literature—a single cystic adenocarcinoma which, according to Eddy *et al.* (*26*), was observed by Habermann in 1 of 503 untreated hamsters, and mammary "carcinosarcomas" found in 2 of 25 hamsters carrying pellets of methylcholanthrene implanted in the gallbladder fundus and pellets of estradiol implanted subcutaneously (*27*). Hormonal control of mammary glands and of mammary-tumor induction in the hamster has not been studied, but such studies should be illuminating.

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[6] Since completion of this paper, 3 male hamsters transplanted with the 39th transfer of the renal tumor showed no transplant growth during 400 days. Implantation of stilbestrol pellets was followed by readily palpable transplants within 30 days. One of the 3 died after 66 days of stilbestrol treatment; its transplant weighed 20 gm., the remaining carcass, 90 gm. A transplant in a single female treated similarly has not responded to stilbestrol during 75 days. It is concluded that in the male hosts endogenous estrogen may have maintained the tumors in a dormant state prior to exogenous estrogen treatment. Further studies are in progress.

In discussing the long latent period of the subcutaneously implanted tumor and its subsequent development into an actively growing malignant lesion, Horning (*8*) remarked that the latent period could be shortened considerably by the administration of an increased dose of stilbestrol. While I have no data directly related to this suggestion, my data concerning the effect of increased dosage on the latent period for the induction of primary lesions (table 5) are perhaps pertinent. As recorded, no clear-cut differences were observed after the administration of one or five 20-mg. pellets of stilbestrol in ovariectomized hamsters. The observations recorded in tables 5 and 6 suggest strongly that the "potency" and the availability of the estrogen used markedly affect the duration of the latent period. How much of this difference is actually attributable to latent period for induction (initiation?) and how much to variation in growth rate after induction (promotion?) is entirely unknown.

It is interesting that testosterone propionate, progesterone, and deoxycorticosterone acetate were effective in inhibiting tumor induction by stilbestrol (table 8), but not in preventing transplant growth in stilbestrol-treated hosts (table 13). The average growth rate of the transplanted tumor subjected to progesterone inhibition was 16 mg. per day, as compared with 287 mg. per day with testosterone propionate and 68 mg. per day with DCA, which suggests that the clinical use of progesterone in cases of inoperable, metastatic renal carcinomas, or in cases of recurrence following operation, would be justified.

The androgen appeared actually to stimulate transplant growth—287 mg. per day as compared with 126 mg. per day with stilbestrol alone. The argument that this stimulation might be due to the conversion of testosterone propionate into estrogen should apply equally well to its activity in tumor induction; however, since the androgen prevented tumor induction (*10, 11, 18, 28*), its activity here is independent of any possible conversion into estrogen. It has been postulated (*28*) that if androgen is converted into estrogen *in vivo* in the hamster, as in man (*29*), it should be possible to induce renal neoplasia by prolonged treatment with the male sex hormone alone. Many of our male and gonadectomized hamsters have been treated continuously with testosterone propionate for long periods, several of them well over 900 days, with an average daily absorption of 0.2 mg., but none have developed renal tumors. It must be concluded that this amount of androgen does not provide enough converted estrogen for renal tumorigenesis during the normal lifespan of the average hamster. It is possible that increasing the daily absorption, *e.g.*, by implanting five instead of one 30-mg. pellet at a time, might provide sufficient estrogen for tumorigenesis. This experiment has been started and, if successful, will be reported at a later time.

A weakness in comparison of latent periods and rates of tumor growth lies in the possibility that each primary tumor may be different in these respects and probably does not represent a uniform tumor-cell population. To minimize this weakness somewhat, in our transplantation work, we try to start with metastases whenever possible, rather than with primary lesions, on the assumption that a metastasis might present a more uniform cell population than its parent tumor. This problem is discussed by Axelrad and Klein (*30*), and others. Another difficulty is the detection of possible variations in growth rate of a single tumor mass at different times, *e.g.*, Robbins (*31*) has photographs of renal tumors showing (on the kidney surface) only slight increase in size over 127 days of stilbestrol treatment, *i.e.*, from laparotomy at 220 days of treatment to autopsy 127 days later. This and other observations suggest a possible lack of uniformity in the rate of tumor growth.

The shortest latent period for detectable transplant growth and tumor induction was reported by Dontenwill and Eder (*19*) in orchiectomized hosts transplanted subcutaneously with the first passage of a renal tumor and injected intramuscularly with 0.6 mg. of diethyldioxystilbene, or its dipropionate, every 2d day. Both subcutaneous and intraperitoneal tumor growth were observed after 14 days.

Horning (*6*) reported that unilateral nephrectomy shortened the latent period for tumor induction from 287 ±24 days in non-nephrectomized animals to 190 ±29 days in unilaterally nephrectomized animals. He used no transplants in this study, but in other papers (*7*, *28*) he reported a progressive shortening of the latent period in successive serial transfers of the tumor.

Ising (*20*) removed the right ureter from 12 male hamsters and treated them as usual with stilbestrol. During 280 days, multiple tumors developed in all kidneys, but were consistently and markedly larger on the operated sides. Although Ising supplied no direct information on the latent period, it would appear from her work that ureterectomy either shortens the period or accelerates tumor growth rate after induction. Unilateral ureteral ligation was also performed by Robbins (*31*) who observed no accelerated tumor growth on the side operated on, nor any indication of increased tumor growth in the atrophic kidney after unilateral ligation of the renal pedicle.

That kidney stimulation through unilateral nephrectomy (Horning) and kidney atrophy (Ising) should have similar effects on the latent period for induction, or on the growth rate of tumors after induction, has no obvious explanation. Learning of Horning's work, we unilaterally nephrectomized 11 males and implanted them subpannicularly with 20-mg. pellets of stilbestrol, as described. Three died less than 150 days later without acquiring tumors; the remaining 8 were examined for renal tumors at laparotomy after 150 and 200 days of treatment and killed after 231 days, as presented in table 7. For purposes of comparison this table includes the non-nephrectomized, but stilbestrol-treated, males whose periods of treatment approximated those of the nephrectomized group; these 12 animals were not run simultaneously as a control group, but were taken from records already available. What difference there is between the characteristics of renal tumors in the two groups is in favor of the controls. Our results, therefore, do not parallel those obtained by Horning.

Horning observed no evidence of compensatory hypertrophy in the remaining kidneys of his unilaterally nephrectomized animals (*17*). Our series was like his in this respect—the average weight of the right kidney in the experimental group was 244 mg. as compared with an average weight of 288 mg. in the controls. Presumably the hamster resembles other mammals in that it is capable of compensatory renal hypertrophy. If so, it is likely also that the apparent inhibition by estrogen (*32*) of the mitosis-stimulating factor in the serum of unilaterally nephrectomized rats (*33*) occurs in the hamster, too. Although Horning (*6*) refers to 12 unilaterally nephrectomized, but otherwise untreated, hamsters, no data are included; he states (*17*), however, that they, too, showed no evidence of compensatory hypertrophy. Unfortunately my experiment did not include this important control group. It can be stated that compensatory renal hypertrophy does not occur in the estrogen-treated hamster and that it may not occur in the untreated hamster.

That renal trauma is not without significance in renal neoplasia is suggested by certain observations. For example, tumor tissue was induced in fragments of kidney transplanted from newborn male and female hamsters. No transplant growth occurred, the fragments being reduced to tiny cysts; in spite of this severe involution, microscopic tumors developed (*10, 34*). Also, after renal traumatization caused by deliberate needle punctures (*35*), the administration of stilbestrol for 200 days resulted in the appearance of tumor tissue along each scar. In 8 of the animals used, other microscopic tumor foci were found in the punctured kidneys and in 10 of the intact kidneys; however, the consistent presence of tumor tissue along each puncture line suggests that traumatization facilitates tumor induction. Since no tumors developed in 11 control males that did not receive stilbestrol after a similar kidney injury, it may be concluded that the stilbestrol acted as a chronic stimulus to tumor induction in cells bordering the puncture lines. Comparable observations were made by Horning (*6*) who found particularly large, rapidly growing tumors developing from residual pieces of kidney tissue accidentally left during unilateral nephrectomies in male hamsters treated subsequently with stilbestrol.

It would appear that the latent period for tumor induction and the rate of tumor growth vary with the condition of the experimental animal. In view of the intrinsic growth potential of the renal tumor cell, as indicated by its growth in tissue culture [(*36*, *37*), and fig. 26], and on the chick chorio-allantoic membrane (*38*), this variation might be attributed to corresponding variations in substances inhibiting the growth of the tumor cell. Why unilateral nephrectomy, renal trauma, or the manner of estrogen administration should affect the production or the activity of such inhibitors of tumor induction and tumor growth is unknown. Evidence favoring the existence of these inhibitors is that the growth of the autonomous variants of the renal tumor, in intact hosts receiving no exogenous estrogen, is greatly reduced over that characteristic of the dependent tumor transplant in estrogen-treated hosts (unpublished data). Cell lines protected from the restraining influence of such normal mechanisms, for a variable number of generations, might evolve into types requiring modified or, eventually, entirely different mechanisms for their control from those effective on their original parent cells; this, of course, would correspond to the acquisition of full autonomy.

When we observe the growth of transplants, as opposed to tumor induction, the role of antibodies should, of course, receive careful consideration, unless it can be established that the host does not react to the tumor cell as to a foreign protein. That over-all differences in protein structure do occur in the renal tumor, as compared with normal hamster kidney, has been established by Easty and Ambrose (*39*); the tumor proteins are characterized by a more random structure which should facilitate attack by hydrolytic and other agents. Weiler (*40*) has described antigenic differences between the renal tumor and normal hamster kidney.

The latent period for transplant growth, although probably affected by the same factors involved in tumor induction, is actually quite another matter, for here we are concerned not with tumor induction from normal cells but, first, with the initiation and maintenance of growth, or with the liberation from growth restraint, on the part of tumor cells already present and, second, with multiple changes in the character of the tumor cells during successive passages as they metamorphose from an estrogen-dependent to an autonomous state.

That the action of estrogen is directly on the kidney is indicated by the failure of estradiol to induce tumors when applied in the path of the hepatic portal drainage, and by the ability of stilbestrol to induce the tumors in the hypophysectomized male hamster (*10*). If the renal-tumor cell utilizes the estrogen as a nutrient, it should be reflected in a decreased recovery of estrogen metabolites in the urine; studies of stilbestrol metabolism offer little support for this concept (*41*). It would appear reasonable, then, to assume tentatively that the initial effect of estrogen on renal-tumor induction, and on the early support of neoplastic growth, is through interference with the action of the substances which normally control the growth of renal tissues. Since the majority of kidney cells continue growth at a rate no higher than normal, there is probably no interference with the production of the growth inhibitors. It is unlikely that these growth-inhibiting substances are all of gonadal and/or adrenal origin, because if they were, general kidney hyperplasia should follow adrenal extirpation in the gonadectomized, cortisone : estrogen-treated hamster. There is, at present, no evidence that this actually occurs, although, as mentioned earlier, it is difficult to establish total adrenalectomy in this species.[7]

After the removal of estrogen, the renal tumor not only may cease to grow but, within a single week, (*42*, *43*) may show some morphological signs of regression. During the ensuing regression period the relative immunity of the neoplastic cell to normal growth inhibition would appear to be removed. Associated with this removal, and as part of the regression process, indication of "redifferentiation" of tumor cells is sometimes recognizable. For example, although tubule formation does not occur in the healthy neoplastic tissue, it may appear during early stages of tumor regression (fig. 6); very rarely this may go to the extreme of transitional epithelioid formations (figs. 24 and 25). This capacity of "dedifferentiated" renal-tumor tissue to "redifferentiate" during tumor regression, in the hamster, may be related to the capacity of undifferentiated metanephrogenic blastema cells of mice to form tubules when transplanted *in vivo* in the absence of a known kidney inductor, although such tubule formation does not occur *in vitro* unless there is an inducing tissue, *e.g.*, spinal cord (*44*).

Tumor regression is sometimes confused with loss of viability. Gross and histological evidence of regression may be extreme, with no recognizable, intact, tumor cells in the sections, and yet, following suitable stimulation *in situ*, or following transplantation of remnants, when found, into a new host, subsequent growth frequently demonstrates the presence of viable cells within the regressed area. With the renal tumor this may be demonstrated even after a regression of 200 days (table 10), at which time the formerly healthy, compact tumor nodules have been reduced to small, clear vesicles. Similar observations have been made by other investigators using other dependent tumors. This means that even in the primary renal tumor the differences in dependency between different tumor cells are probably considerable, and may mean that, in some instances, a primary nodule may even contain cells with partial or complete autonomy, as evidenced (1) by the growth of metastases, from primary renal tumors, transplanted into stilbestrol-treated female hosts (table 14, and figs. 13 and 22) and (2) by the occasional finding of histologically healthy tumor cells in what at autopsy appeared as tiny scars left from completely regressed primary tumor nodules (fig. 23). The implications of these observations are considerable, *e.g.*, the time and rate at which a hormonally induced neoplasm changes from hormone dependency to autonomy might well be determined chiefly by the ratio between the number of 100 percent dependent and the number of partially or completely autonomous cells in competition with each other for survival.

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[7] It has been demonstrated (Algard, F. T.: Hormone-induced tumors. I. Hamster flank organ and kidney tumors *in vitro*. J. Nat. Cancer Inst. In press.) that growth and survival of the renal tumor in tissue culture are independent of estrogen in the media, but that in organ culture growth does not occur and survival time is greatly reduced in the absence of estrogen. The implications of these observations with reference to the mechanism of estrogen action in renal tumorigenesis are very significant.

The moment when a tumorigenic stimulus is applied is of importance when endocrine or other metabolic functions of a rhythmic nature are involved, as in the induction of kidney tumors in female hamsters. The total duration of exposure to such stimuli varies greatly in importance with different tumors, *e.g.*, a single brief application may suffice with chemical carcinogens, whereas continuous application is necessary with estrogen-induced tumors. The one achieves autonomy quickly, the other only after a prolonged dependent phase. The important difference appears to be the rate of change from normalcy to autonomy. This emphasizes the value of hormonally induced tumors in the study of mechanisms of carcinogenesis. That this value is not as widely recognized as it deserves is stressed by Dodds (*45*).

CONCLUSIONS

(1) Renal-tumor induction by stilbestrol is inhibited and usually prevented by testosterone propionate, progesterone, or deoxycorticosterone acetate.

(2) In renal-tumor induction, dosage with a given estrogen, implanted subpannicularly, appears to involve more than daily absorption rate.

(3) Little, if any, significant differences in the size, number, or latent period of primary renal tumors occur in ovariectomized hamsters implanted subpannicularly with one or five 20-mg. pellets of stilbestrol.

(4) The latent periods for tumor induction required by estrone, estriol, ethynyl estradiol, and 7-methylbisdehydrodoisynolic acid (Fenocylin) are much longer than for stilbestrol and estradiol.

(5) In the estrogen-treated hamster no compensatory renal hypertrophy follows unilateral nephrectomy. This operation appears to have no significant effect upon the latent period for tumor induction in the remaining kidney.

(6) Renal traumatization appears to facilitate the induction of renal tumors.

(7) Renal tumors may be induced in kidney fragments, from newborn male or female donors, transplanted subpannicularly in stilbestrol-treated male hamsters.

(8) In the induction of renal tumors, estrogen probably acts directly upon kidney tissue, since estradiol pellets implanted in the path of the hepatic portal circulation are not effective and since the tumors may be induced in hypophysectomized hamsters.

(9) The estrogen-induced renal tumor is transplantable as an estrogen-dependent neoplasm.

(10) When the tumor is transplanted without exogenous estrogen, it does not grow until after several serial passages supported by estrogen.

(11) Transplant growth is inhibited, but not stopped, by the addition of progesterone or deoxycorticosterone acetate to the stilbestrol used to support tumor growth.

(12) The clinical trial of progesterone in cases of inoperable, metastatic, renal carcinoma is indicated.

(13) Transplant, or primary, tumor growth may be stimulated by the addition of testosterone propionate to stilbestrol.

(14) The stimulation of renal-tumor growth by testosterone propionate is not due entirely to conversion of the androgen into estrogen.

(15) After the eleventh passage and possibly earlier, but not as early as the first passage, transplant growth may be maintained by testosterone propionate alone.

(16) Subpannicular renal-tumor transplants show higher average growth rates than intrathoracic or cheek-pouch transplants.

(17) After the third passage, at least, 100 percent growth may be expected in subpannicular renal-tumor transplants supported by stilbestrol.

(18) The latent period for transplant palpability and the survival time of the host tend to become shorter, and the rate of transplant growth greater, with succeeding serial passages.

(19) In calculating survival time it is important that *all* experimental animals involved live as long as possible.

(20) It is likely that the growth rate of both the primary and transplanted renal tumor is not constant over a long period, *i.e.*, there is probably a considerable range from the calculated mean daily growth rates.

(21) In our series, the acquisition of autonomy by the transplant has started early and has been slowly progressive.

(22) Variations in the degree of dependency, or autonomy, characterize different cells within a single tumor nodule.

(23) Transplants may metastasize as early as 63 days, primarily to the lungs, the adjacent lymph nodes and, less frequently, to the mesenteries, diaphragm, spleen, liver, and heart.

(24) Although the renal tumor may be induced in females only under conditions of low progesterone secretion, it can be transplanted successfully, even in the first serial passage, to stilbestrol-treated, female hosts.

(25) Ordinarily the primary tumor and/or its transplants undergo marked regression after the removal of stilbestrol, but exceptions sometimes occur with transplants, *e.g.*, in our tenth, twelfth, and fourteenth passages.

(26) Even 200 days after the cessation of stilbestrol treatment, latent viability may be demonstrated in regressed renal tumors by resumption of estrogen treatment.

(27) The latent period for tumor induction and the latent period for transplant palpability do not necessarily coincide.

(28) Cortisone increases the incidence of primary renal tumors and of metastases in stilbestrol-treated, renal-tumor-transplant-bearing, male hosts.

(29) Cortisone alone will not maintain transplant growth in castrated males or females, or in adrenalectomized, spayed hamsters.

(30) Stilbestrol will maintain transplant growth in the cortisone-treated, adrenalectomized, male hamster.

(31) In the transition from dependency to autonomy, the renal tumor progresses through various stages of limited autonomy.

(32) Competition for survival between hormone-dependent and hormone-independent tumor cells may start as early as the first serial transplant in affecting the time and rate at which the transition to complete autonomy occurs.

(33) The essentially normal growth behavior of the majority of kidney cells under chronic estrogen treatment suggests either that only a minority of renal cells are capable of neoplasia under these conditions or that local interference with the production, or efficacy, of inhibitory factors in their environment occurs.

(34) Cancerous tissue differs from normal in that it has escaped the mechanisms which restrict the growth of the normal tissue from which it arose. This escape may be complete (autonomy) or incomplete (partial autonomy). It is during the transition from a normal to an autonomous state that mechanisms of neoplasia can best be studied.

(35) The transition from normalcy to autonomy, through a prolonged dependent phase, makes hormonally induced neoplasia especially favorable for studies of the mechanisms of carcinogenesis. This fact deserves wider recognition.

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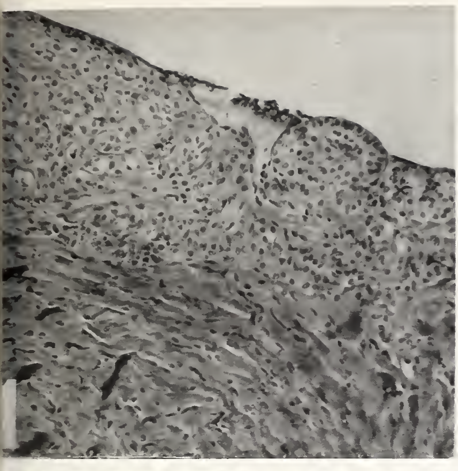
PLATE 1

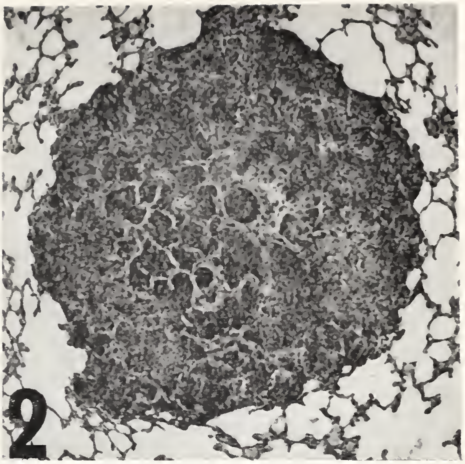
FIGURE 1.—Renal-tumor tissue from first serial transfer, injected intrathoracically, and growing in the pericardium at apex of the heart. × 110

FIGURE 2.—Pulmonary metastasis from twelfth serial transfer of a primary renal-tumor metastasis. A 272-day-old male autopsied after 150 days of treatment with stilbestrol and transplant. The host kidneys contained no tumors. × 50

FIGURE 3.—Metastasis, in a cervical lymph node, from eleventh serial subpannicular transfer of a renal-tumor metastasis. The male host received stilbestrol and cortisone for 167 days. [Reprinted by permission of *Cancer*, *see* figure 6 (*10*).] × 440

FIGURE 4.—Cellular vacuolization, indicative of early regression, in a primary tumor of left kidney in a male treated with stilbestrol for 325 days; animal was killed 14 days after removal of stilbestrol pellets. × 220

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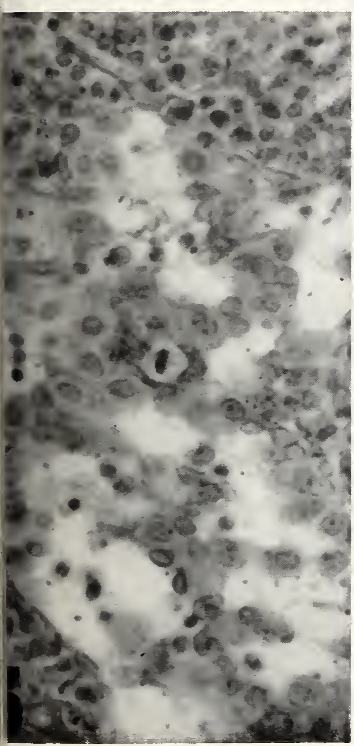

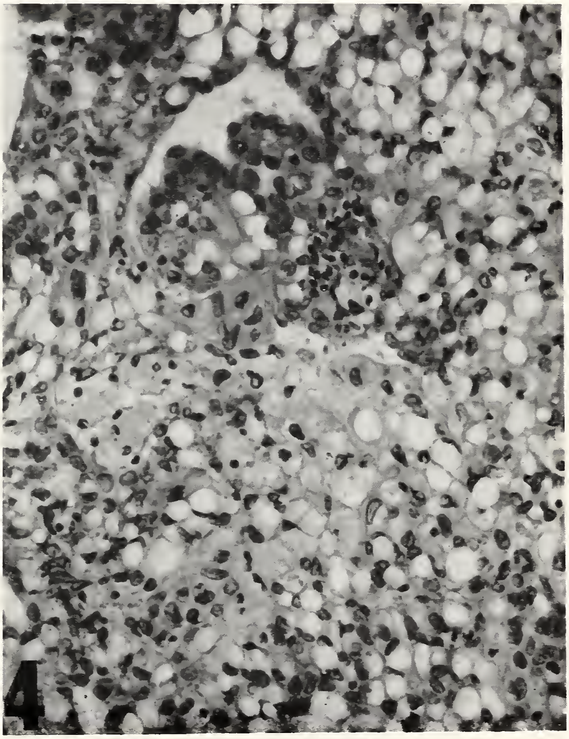
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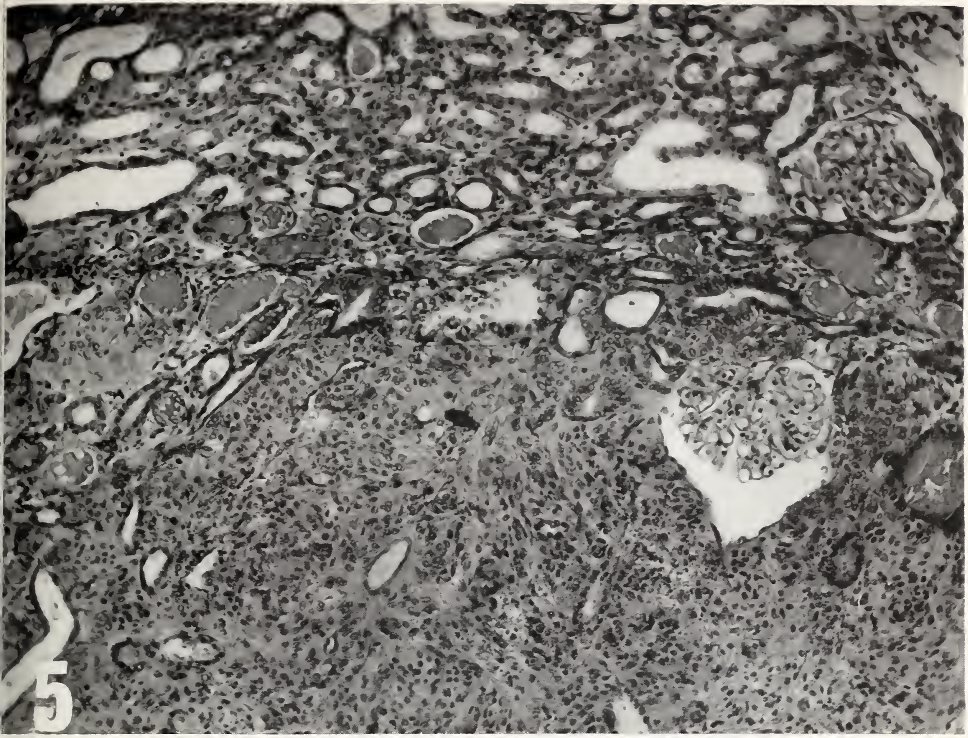
Plate 2

Figure 5.—Primary tumor in left kidney of a male hamster implanted subpannicularly with 20- and 30-mg. pellets, respectively, of stilbestrol and testosterone propionate on the 116th day of life; treatment continued for 359 days. This was the only one of 56 males treated in this way that acquired renal tumors. × 120

Figure 6.—Suggestion of tubule formation in a partially regressing primary renal tumor in right kidney of a male implanted subpannicularly with a 20-mg. pellet of stilbestrol on the 50th, and again on the 200th day of life; stilbestrol removed after 300 days of treatment and animal killed 7 days later. × 440



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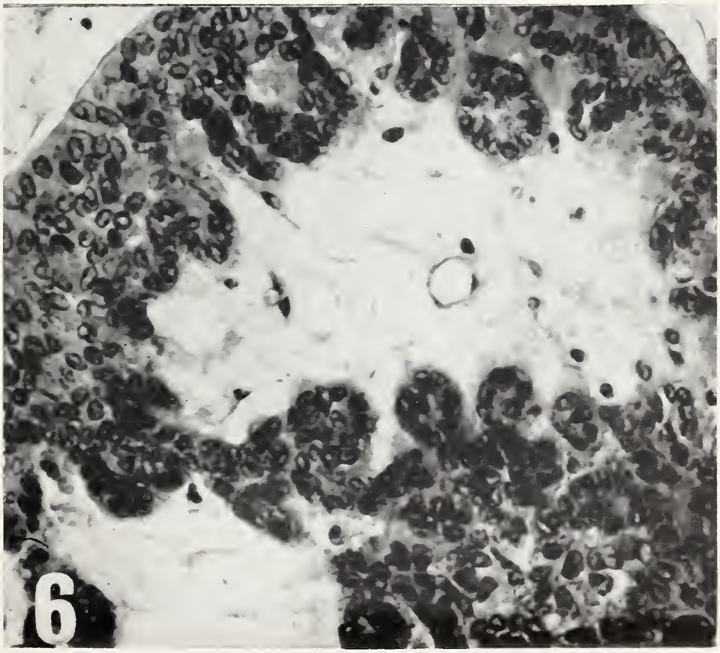

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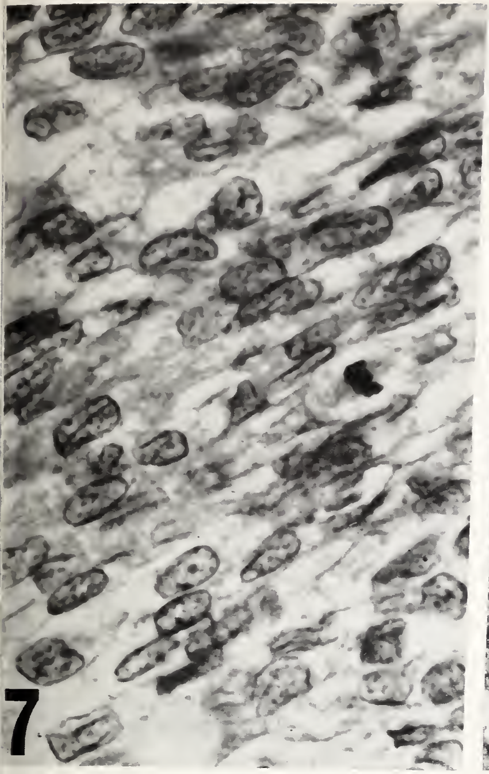
PLATE 3

FIGURE 7.—Primary tumor in left kidney of a male implanted subpannicularly with a stilbestrol pellet on the 52d day of life; treatment continued until animal was killed 435 days later. On the 305th day a pellet of testosterone propionate was added to the estrogen and treatment continued until death, 182 days later. Healthy primary tumor nodules and metastases were abundant. × 920

FIGURE 8.—Metastases from primary renal tumors of males implanted subpannicularly with stilbestrol pellets on the 50th day of life; treatment continued until animals were killed 480 (fig. 8A) and 506 (fig. 8B) days later. On the 303d day pellets of testosterone propionate were added to the estrogen; treatment continued until death of animals, 227 (fig. 8A) and 253 (fig. 8B) days later. (A) mesentery, × 220; (B) cervical lymph node. × 160



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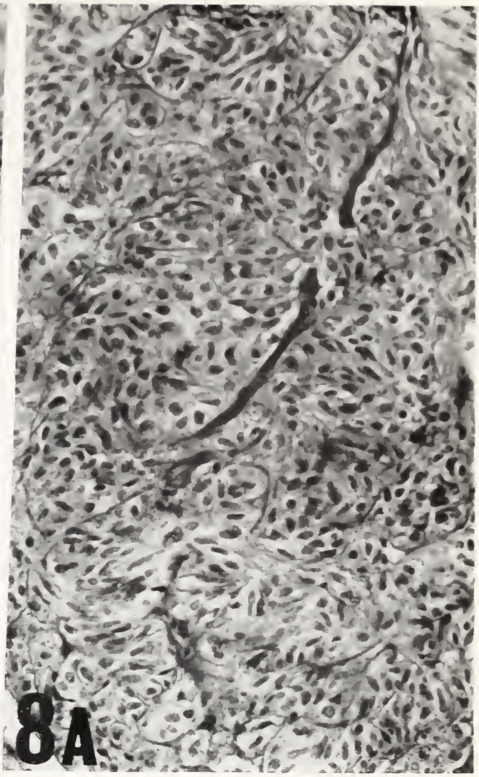

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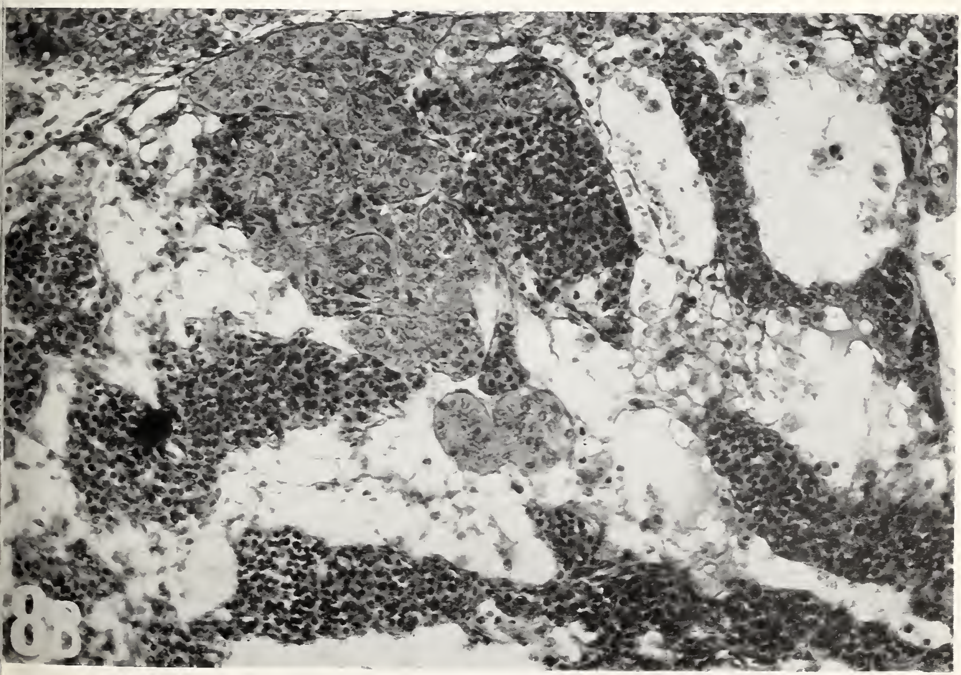


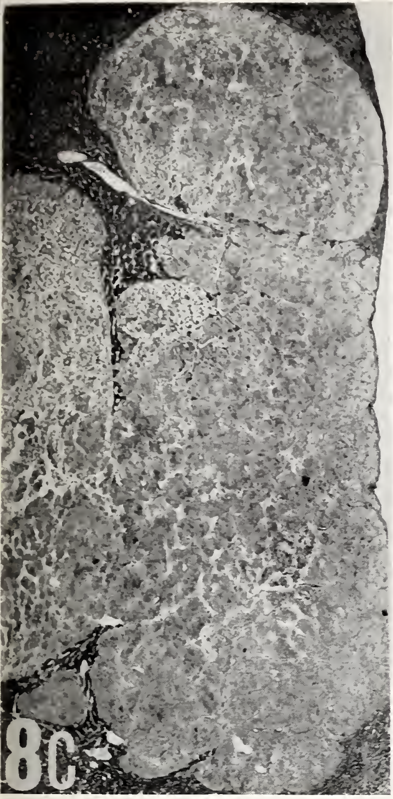
8B

PLATE 4

FIGURE 8C.—Same as figure 8B, except that metastases were in liver. [Reprinted by permission of *Cancer, see* figure 4 (*10*).] × 20

FIGURE 9.—Healthy tumor cells found in otherwise necrotic renal-tumor transplant of the tenth serial passage. The orchiectomized host had received stilbestrol for 65 days, starting at the time of transplantation. The transplant was palpable after 65 days at which time the estrogen pellet was removed, and the animal was killed 200 days later; the transplant was no longer palpable, but was recovered at autopsy and weighed 7.4 mg. × 800

FIGURE 10.—Healthy pulmonary metastases from the sixth subpannicular passage of a renal tumor in a male host, treated with stilbestrol for 175 days and deprived of it for 122 days before death. × 47



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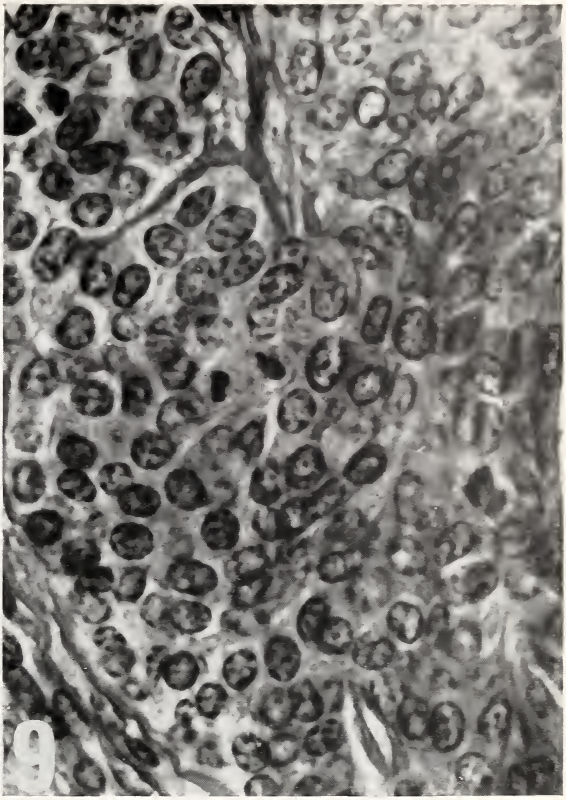

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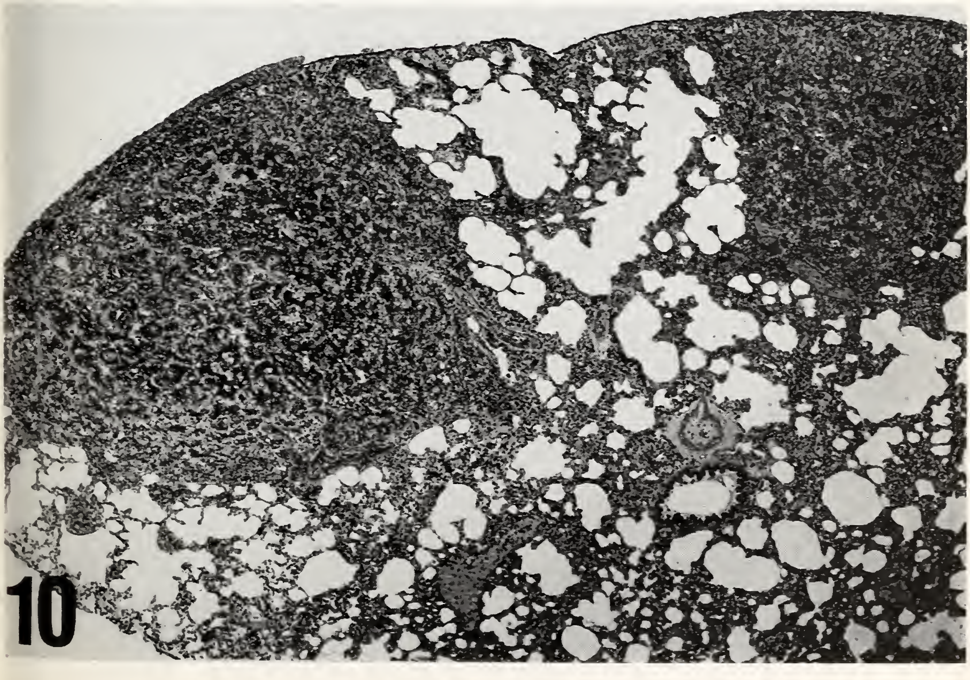

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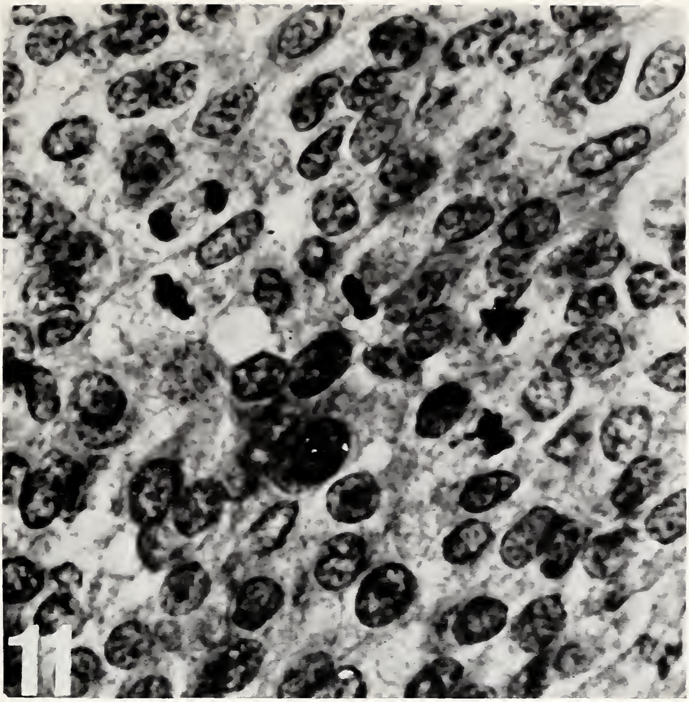
PLATE 5

FIGURE 11.—Seventh serial transfer of a renal-tumor metastasis. The male host carried the transfer and stilbestrol from the 31st day of life until killed 195 days later. The transplant was palpable after 51 days at which time a pellet of testosterone propionate was implanted and left until animal was killed 144 days later. Transplant weighed 32 gm. and remaining carcass 116 gm. No regression was evident. [Reprinted by permission of *Cancer, see* figure 9 (*10*).] × 920

FIGURE 12A.—Twelfth serial transfer of a renal-tumor metastasis. Male host carried the transplant and a pellet of testosterone propionate from the 122d day of life until killed 150 days later. The transplant weighed 394 mg. and contained many dividing cells. × 400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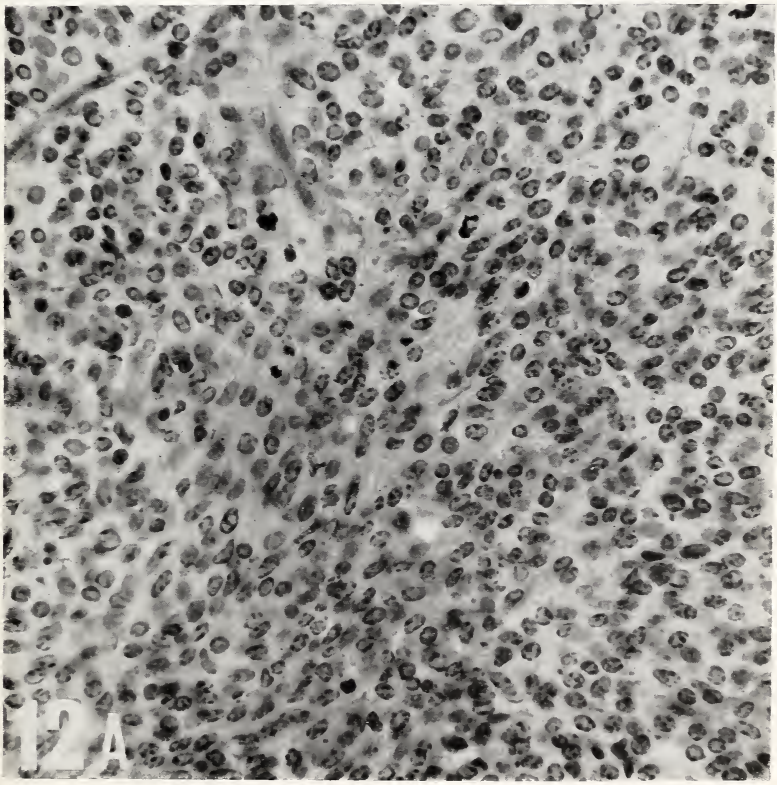
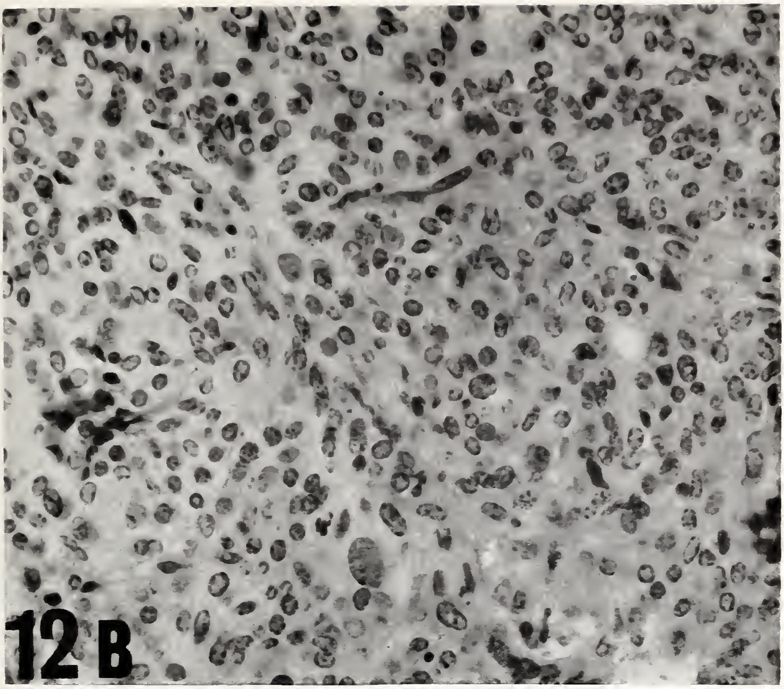

12 A

PLATE 6

FIGURE 12B.—Same as figure 12A. Variations in nuclear size.

FIGURE 13.—First serial transfer, from a primary renal-tumor metastasis, transplanted subpannicularly in an intact female host on the 136th day of life and continued for 236 days. The host received a stilbestrol pellet at the time of transplantation. × 200

FIGURE 14.—Pulmonary metastasis in a male hamster treated with stilbestrol, cortisone, and the eleventh serial transfer of a renal-tumor metastasis from the 51st day of life until killed 179 days later. Primary tumors occurred in each kidney and the transplant weighed 81 gm. as compared with a carcass weight of 77 gm. × 32



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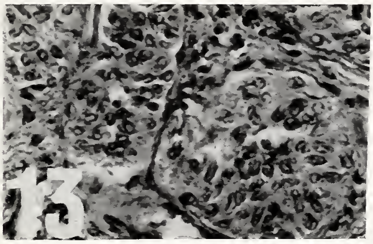

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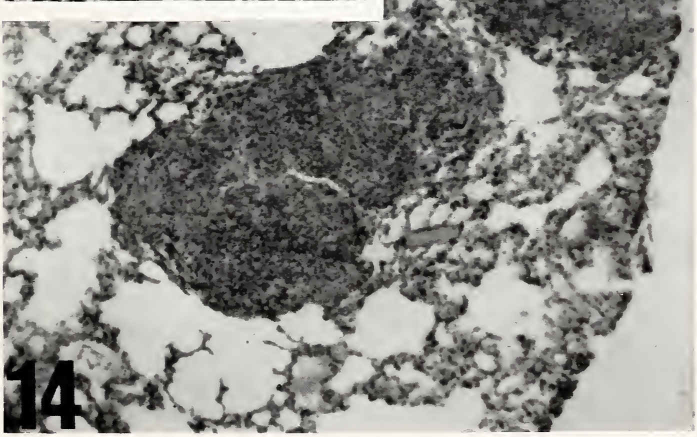


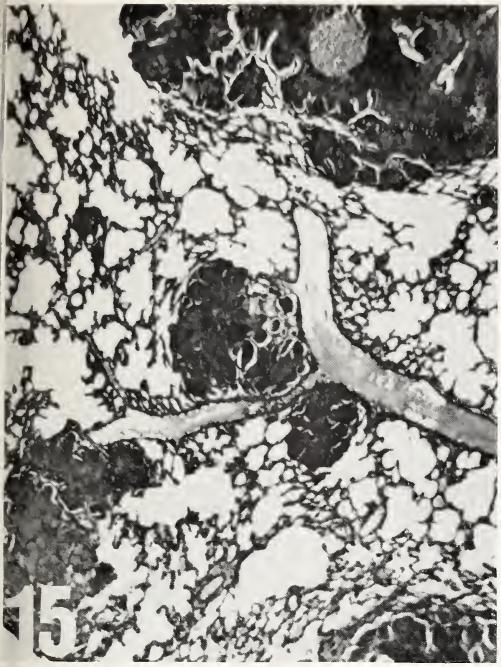
14

Plate 7

Figure 15.—Same as figure 14, but treated for 175 days. Primary tumors occurred in right kidney and the transplant weighed 38 gm. as compared with a carcass weight of 66 gm. × 16

Figure 16.—Transplant regression in an ovariectomized host treated with stilbestrol for 68 days, after which the estrogen was replaced by cortisone and adrenal glands were removed. The animal was killed 75 days later. (A) × 16; (B) × 280

Figure 17.—Portion of the first serial passage of a renal-tumor metastasis, in an orchiectomized hamster, maintained on stilbestrol for 180 days, after which estrogen was replaced by cortisone for 100 days. Marked regression occurred, but many healthy cells remained. × 280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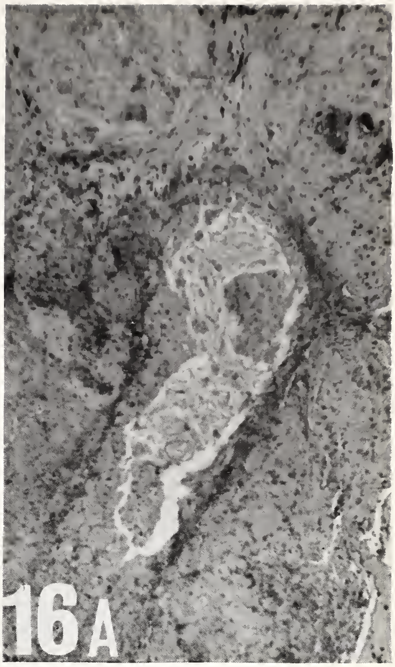
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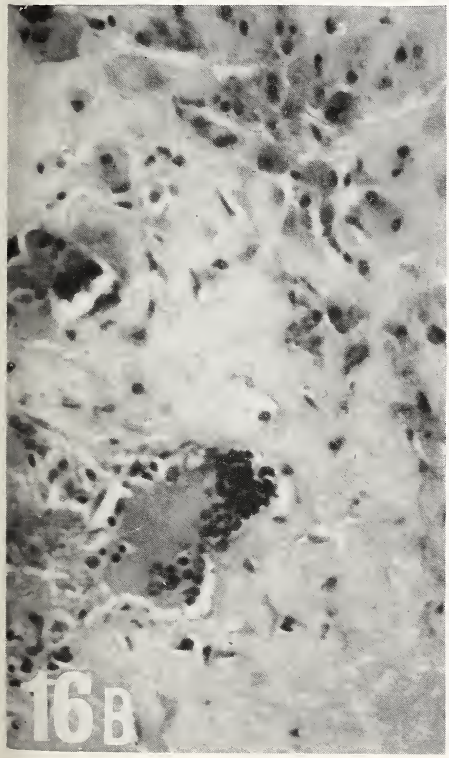
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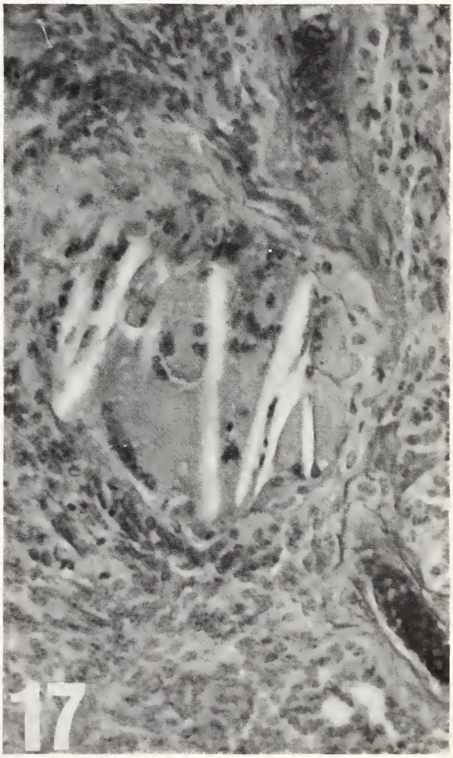
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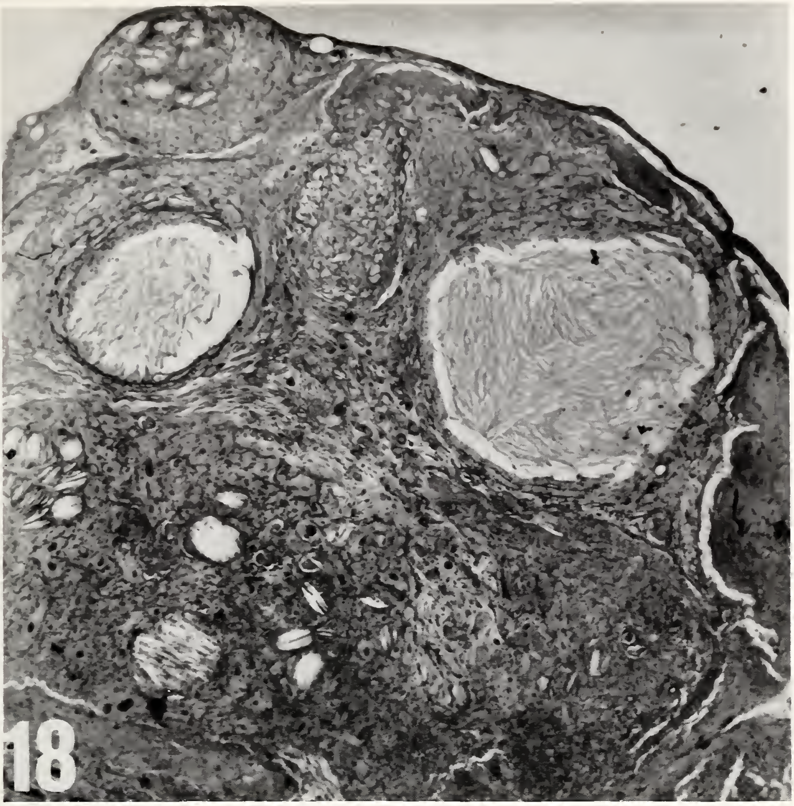
Plate 8

Figure 18.—Same as figure 17. × 20

Figure 19.—Healthy tumor cells in the twelfth serial transfer of a renal-tumor metastasis maintained in a stilbestrol : cortisone-treated, adrenalectomized castrate for 53 days. × 1,000

Figure 20.—Solid, cystic, and multiloculated, papillary types of tumors in right kidney of a male implanted subpannicularly with five 20-mg. pellets of stilbestrol : cholestrol (1:4) on the 49th day of life and killed 424 days later. × 7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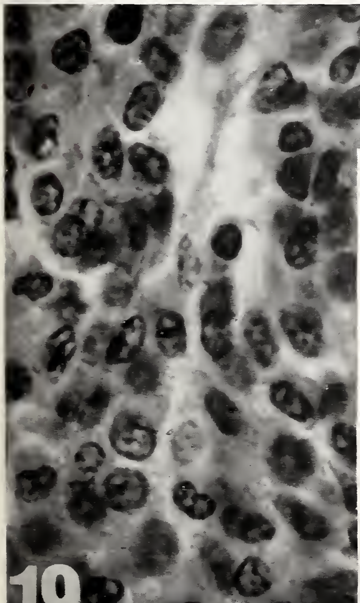
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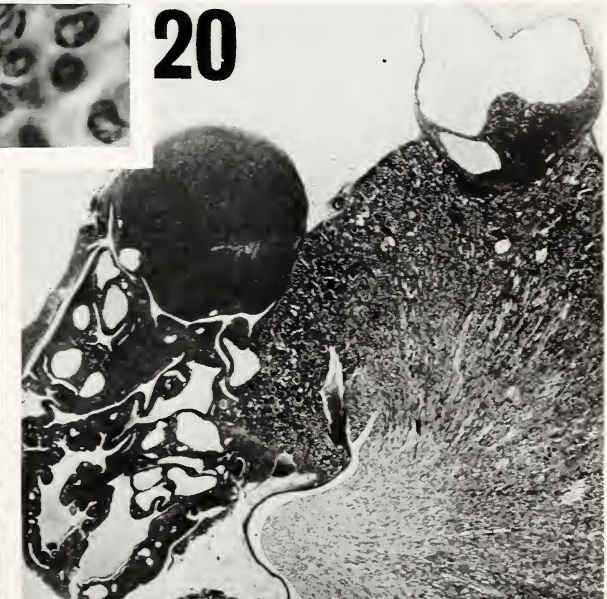
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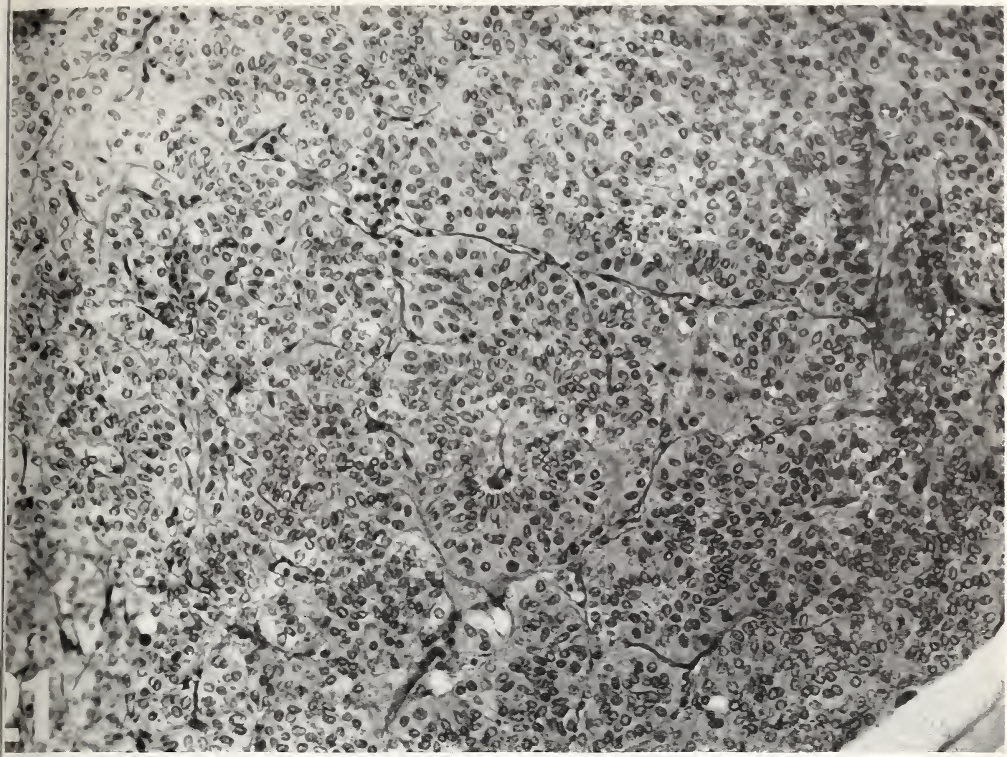
PLATE 9

FIGURE 21.—Treatment same as in figure 20. Mesenterial metastasis from primary renal tumor. × 160

FIGURE 22.—First serial transplant from a primary renal-tumor metastasis, transferred subpannicularly in an intact female host on the 123d day of life and continued for 149 days. The host received a stilbestrol pellet on the 109th day and was killed 163 days later. × 200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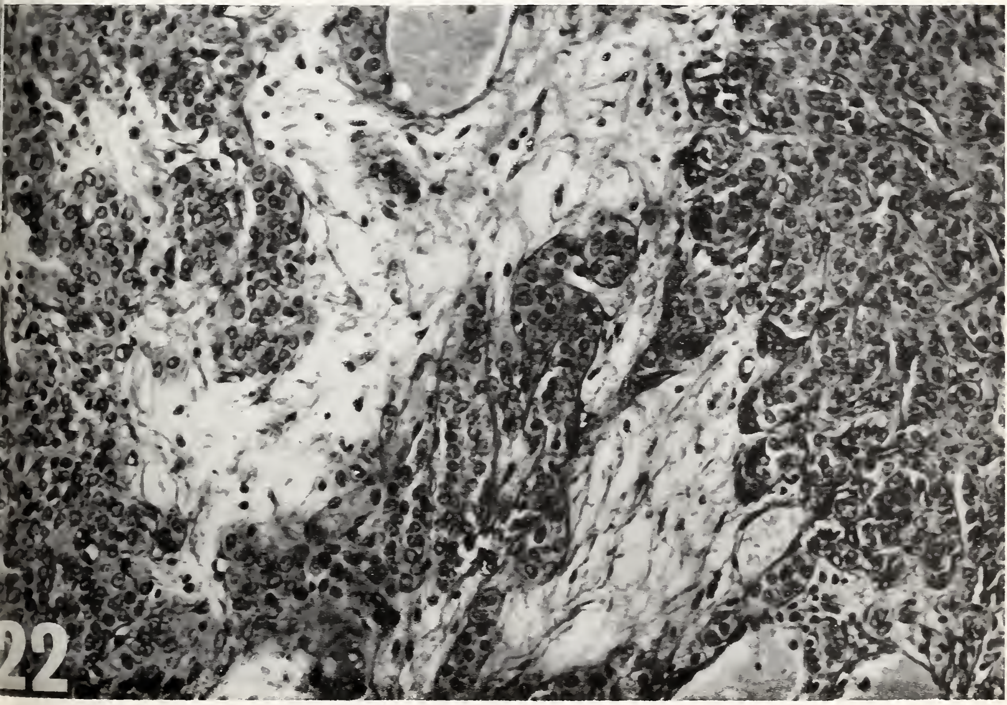
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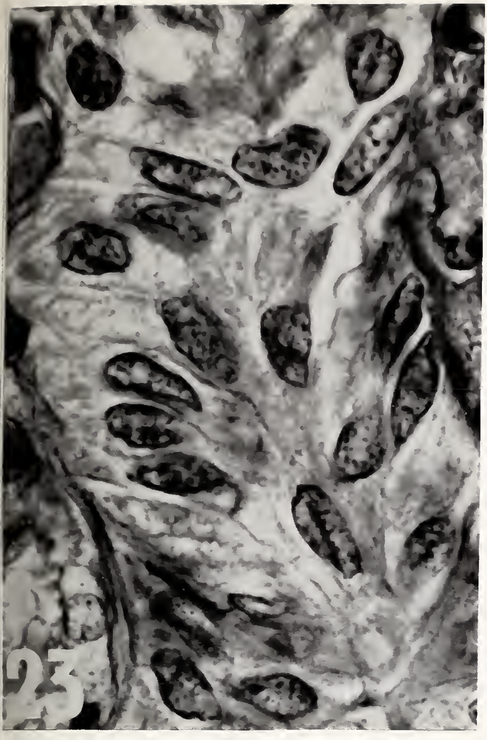
Plate 10

Figure 23.—Healthy primary tumor cells in what appeared at autopsy as a tiny scar in left kidney of male treated with stilbestrol for 253 days, after which time the stilbestrol pellet was removed and tumor nodules in the kidney confirmed at laparotomy. Sixty days later there were still tiny tumor nodules, but they were amber-colored. Animal died after another 67 days—127 days after removal of stilbestrol. At autopsy the only recognizable tumor remnant was a single scar. × 1,300

Figure 24.—Early regression, and transitional epithelium formation, in a subpannicularly transplanted renal-tumor metastasis of the 13th serial passage. Host orchiectomized at 100 days; transplanted with tumor and implanted with stilbestrol pellet at 110 days; with cortisone pellet at 185 days; stilbestrol pellet and adrenal glands removed at 192 days; animal died at 201 days of age. Transplant weighed 109 mg. × 250

Figure 25.—Same as figure 24, but × 740

Figure 26.—Subpannicularly transplanted explant of eighth serial passage of a renal tumor. Donor for the explant was a hypophysectomized male treated with stilbestrol for 144 days and with the explant for 137 days. The host for the explant was a male treated with stilbestrol for 361 days and with the explant for 358 days. The tumor was cultured by Dr. F. T. Algard. × 200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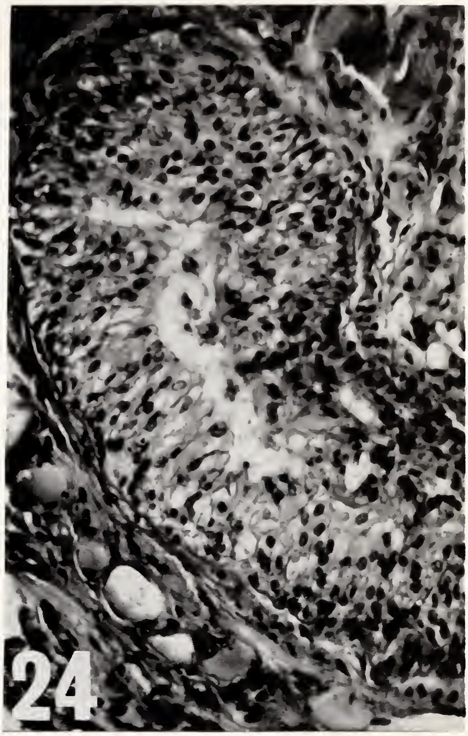
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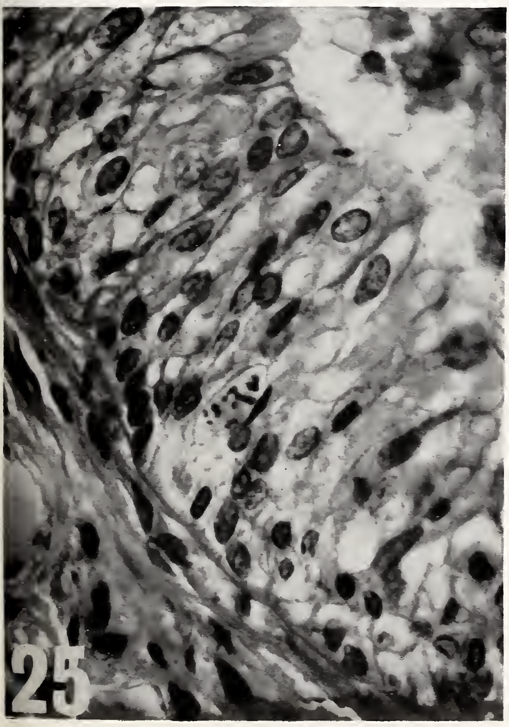
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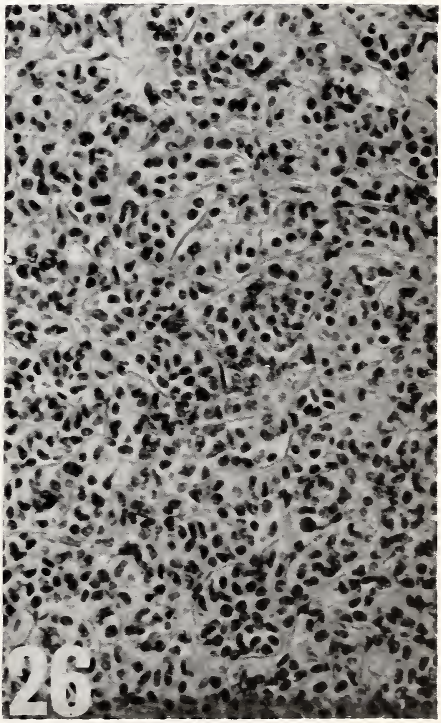
26

Estrogen-Induced Tumors of the Kidney. IV. Incidence in Female Syrian Hamsters [1,2,3]

HADLEY KIRKMAN,[4] *Department of Anatomy, Stanford University School of Medicine, Stanford, California, and the Chester Beatty Research Institute, Institute of Cancer Research, Royal Cancer Hospital, London, England*

SUMMARY

Continuous administration of estrogens to Syrian hamsters resulted in the appearance of malignant renal tumors in intact males and gonadectomized males and females, but not in intact females. Since administration of progesterone with diethylstilbestrol prevented induction of renal tumors, it was suspected that progesterone might be responsible for the failure of intact females to acquire tumors. In addition to ovariectomy, 4 methods of inducing tumors in females were attempted. All were based on starting treatment at times of little or no progesterone secretion. Each method was successful. The methods were: (1) starting treatment with estradiol and diethylstilbestrol during metestrum; (2) starting treatment with diethylstilbestrol on the 2d, 10th, or 18th day of life; (3) administering testosterone propionate on the 1st day of life and replacing it with diethylstilbestrol on the 50th or 196th day of life; (4) transplanting kidney tissue from newborn female donors to diethylstilbestrol-treated male hosts. Experimental results justified these conclusions: Renal tumors may be induced in female hamsters by starting estrogen treatment (1) after removal of the ovaries; (2) at times of lowest progesterone secretion, *i.e.,* late in day 4 of the cycle; (3) before reproductive maturity; (4) in animals whose pituitary glands have been irreversibly "masculinized" by treatment with testosterone propionate at birth; (5) in females maintained in male environments. Continued progesterone secretion ordinarily prevents induction of tumors in estrogen-treated female hamsters. It is, therefore, possible to induce tumors by initiating treatment at times of low progesterone secretion.

THE OCCURRENCE of estrogen-induced renal tumors in male hamsters has been reported in earlier papers (*1–7*) and confirmed by other investi-

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[1] Received for publication December 29, 1958; revised May 25, 1959.

[2] This investigation was supported in part by research grants from the American Cancer Society, Inc., the National Cancer Institute, National Institutes of Health, Public Health Service, and the Jane Coffin Childs Memorial Fund for Medical Research.

[3] The experimental work reported here was completed at Stanford University; the data were assembled and the manuscript prepared during the author's tenure of a Senior National Science Foundation Fellowship (1957–58) and a Special U.S. Public Health Service Fellowship (1958–59) at the Chester Beatty Research Institute. All estrogens and androgens used in these experiments were supplied through the generosity of Ciba Pharmaceutical Products, Inc., Summit, N. J. The ACTH preparations were supplied by the Armour Laboratories, Chicago, Ill., and by Dr. A. P. Rinfret of the University of California School of Medicine.

[4] I wish to express my gratitude to the Jane Coffin Childs Memorial Fund for Medical Research, the American Cancer Society, the National Cancer Institute, the National Science Foundation, the Chester Beatty Research Institute, and the following individuals. Catherine Crismon, Gladys T. Kirkman, Marilyn E. Robbins. Helen S. Thayer, and Doris H. Wurster for valuable assistance rendered at various stages of the work reported here.

gators (*8–16*). The first estrogen-induced renal tumors reported in female hamsters occurred after gonadectomy (*17*). The following year they were noted in two pregnant females (*18*) and in females treated before the first ovulation (*7*). Not until 1957 were they reported, in preliminary fashion, in mature, intact females (*19–21*). In the present paper the striking sex difference in the incidence of estrogen-induced renal tumors will be described and accounted for, and methods for inducing the tumors in females will be reported.

MATERIALS AND METHODS

The hamsters were a mixed group—about half of them were purchased from the West Side Hamstery, Tracy, California, and half were born in our own colony, of parents of heterogeneous origin. In the tables only animals 250 days of age or more were included, except when renal tumors were observed at an earlier age, in which case the range in age started with the youngest tumor-bearing animal and included all animals as old as, or older than, that one.

Two ACTH preparations were used: (1) Armour, lot #L54504, 20 percent gel; 0.5 cc. containing 0.75 units, injected daily; (2) Rinfret's equine preparation, EAPE—pool V, 2 mg. per cc.; 0.5 cc. injected daily. The hormone was kept in the freezer in small gelatine capsules, and only enough removed daily to meet daily requirements.

Other methods were described in (*4*).

RESULTS

As an introduction to data on the occurrence of renal tumors in female hamsters, a brief statement regarding the incidence of such tumors in male hamsters should be helpful. In our experience, and in the experience of other workers, there is a long latent period between the initiation of estrogen treatment and the first appearance of renal tumors. In intact males and in gonadectomized males and females, we expect grossly visible, bilateral, multiple renal tumors in 80 to 100 percent of hamsters treated for 250 days with subpannicularly implanted pellets of diethylstilbestrol (stilbestrol); a single 20-mg. pellet for each animal, every 150 days of treatment. As shown in table 1, in 61 intact, untreated males no renal tumors were observed. In contrast, 74 percent of 268 estrogen-treated males did have renal tumors. This percentage would have been much higher if only animals treated for 250 days or longer had been included. The youngest tumor-bearing animal received estrogen for only 136 days. In 60 untreated castrates (table 2) no renal tumors were observed, but of 83 estrogen-treated castrates, 83 percent had renal tumors. This percentage, too, would have been higher had the castrates treated for 178 and 249 days been excluded from the table.

Table 1.—Occurrence of renal tumors in untreated and in estrogen-treated, intact, male hamsters

	Number of animals in sample	Method of administration	Mean age at autopsy, with range (*days*)	Mean duration of treatment, with range (*days*)	Renal tumors	Metastases
Untreated males	61	—	441 250–985	—	—	—
Stilbestrol-treated males	245	Injected and/or pellets*	362 250–750	306 136–659	177 or 83%	50 or 28% of 177
Estradiol-treated males	15	Pellets	406 337–564	355 286–514	15 or 100%	6 or 40% of 15
Estrone-treated males	8	Pellets	515 423–651	465 372–602	7 or 88%	1 or 14% of 7
Total: estrogen-treated males	268	—	380 250–750	323 136–659	199 or 74%	57 or 29% of 199

*Subpannicular injection of 0.6 mg. every 2d day, in sesame-oil or in saline suspension, followed, in some cases, by 20-mg. pellets implanted subpannicularly; in other cases, only pellets used.

Table 2.—Occurrence of renal tumors in untreated and in estrogen-treated orchiectomized hamsters

	Number of animals in sample	Mode of administration*	Mean duration of estrogen treatment, with range	Mean age when orchiectomized, with range	Mean duration of orchiectomy, with range (*days*)	Mean age at autopsy, with range (*days*)	Mean age when transplanted subpannic. with renal tumor, with range	Mean age when hypophysectomized, with range	Stilbestrol treatment preceded by testosterone propionate from 51–81 days of age	Renal tumors	Metastases
Untreated castrates	60	—	—	54 29–73	323 52–600	377 253–653	—	—	—	—	—
Stilbestrol-treated castrates	15	Pellets	367 248–497	45 39–76	373 258–497	418 297–548	—	—	—	15 or 100%	2 or 13% of 15
	27	Pellets	260 207–410	41 27–60	275 208–460	316 250–489	75 36–175	—	—	14 or 52%	12 or 86% of 14
	4	Pellets	328 309–344	60 60–60	331 312–347	391 372–407	180 172–203	365 357–388	—	3 or 75%	0
	8	Pellets	340 178–375	30 30–30	391 230–426	421 260–456	—	—	+	8 or 100%	5 or 63% of 8
	7	Injection	375 361–380	59 58–61	384 371–390	448 429–468	—	—	—	7 or 100%	0
Estradiol-treated castrates	12	Pellets	278 267–329	31 30–32	306 292–359	336 317–389	—	—	—	12 or 100%	0
Estrone-treated castrates	10	Pellets	409 359–416	50 50–50	409 359–416	459 409–466	—	—	—	10 or 100%	0
Total: estrogen-treated castrates	83		320 178–497	31 27–76	336 208–497	376 250–548	—	—	—	69 or 83%	19 or 28%

*Pellets were 20 mg. in weight, implanted subpannicularly, every 150 days. Subpannicular injections of 0.6 mg. every 2d day, in sesame-oil or in saline suspension.

The response of the female hamster kidney is shown in table 3. Of 56 animals, kidney tumors occurred in 1; they were barely visible at autopsy and poorly developed histologically, but occurred in each kidney (fig. 1).

Assuming that circulating progesterone in the estrogen-treated females might be responsible for the failure of renal tumors to develop, we administered estrogen to a series of ovariectomized hamsters, with results as shown in table 4. Of 41 estrogen-treated, ovariectomized animals, 66 percent acquired tumors (figs. 2, 3, and 4) as opposed to the complete absence of renal tumors in 12 untreated controls. This 66 percent would have been much higher had the spayed animals, treated for 121 to 249 days, been excluded from the table.

Also, estrogen treatment was initiated at known stages of the estrous cycle, with the expectation of renal tumors only in association with corpus luteum regression. As disclosed in table 5, a tumor was observed in the group started during middle metestrum B (*22*).

To test further the assumption that progesterone might inhibit the expression of the renal tumor-inducing property of estrogen in the hamster, pellets of both stilbestrol and progesterone were implanted in the 3 categories of hamsters known to be susceptible to this property, *viz.*, intact males, orchiectomized, and ovariectomized animals. As described in table 6, no kidney tumors appeared in any of 11 intact male, 14 castrated, or 13 spayed hamsters, so treated. Neither did tumors occur in any of the 59 progesterone-treated controls listed in table 7.

With these observations as a background, it was decided to attempt the induction of renal tumors in the kidneys of females by three other methods—all based on the initiation of estrogen treatment under conditions of low progesterone secretion. The first method demanded that treatment be started before reproductive maturity. The second method required that newborn females receive an initial exposure to exogenous androgen, so that their pituitary glands might thereby become irreversibly "masculinized," as reported originally by Pfeiffer for the rat and mouse (*23–25*). After this treatment, estrogen was given at 50 days of age, since it had been demonstrated (table 3) that estrogen administered at this age to intact females was not ordinarily followed by renal tumors. The third method consisted of the subpannicular transplantation of portions of kidneys from newborn females into stilbestrol-treated males (*26*). Renal tumors were obtained in all three groups (tables 8, 9, and figs. 5, 6, 7, 8, 9, 10, and 11).

Since progesterone might be secreted by the adrenal glands, and since deoxycorticosterone of adrenal origin, with or without progesterone, might be implicated in the customary failure of females to acquire renal tumors under estrogen treatment, it would be of interest to look for renal tumors in stilbestrol-treated, cortisone-treated, adrenalectomized females. So far this experiment has been performed only in males (table 10).

For reasons discussed later (page 73) it was considered possible that ACTH might inhibit the tumorigenic activity of estrogen in the female; therefore 4 more males were implanted with stilbestrol pellets and

Table 3.—Absence of renal tumors in untreated and in estrogen-treated, intact, mature, female hamsters

	Number of animals in sample	Estrogen used	Mode of administration	Mean age when treatment started, with range (*days*)	Mean duration of treatment, with range (*days*)	Mean age at autopsy, with range (*days*)	Renal tumors
Untreated females	84	—	—	—	—	450 250–790	0
Estrogen-treated intact females	49	Stilbestrol	Injections and/or pellets*	58 36–141	364 250–487	422 300–537	1†
	6	Estradiol	Pellets	48 48–50	312 241–363	360 289–411	0
	1	Estrone	Pellets	51	345	396	0
Total: estrogen-treated females	56	Estrogens	—	57 36–141	358 241–487	415 289–537	1

*Ten received 0.6 mg. every 2d day, in sesame-oil solution or in saline suspension, injected subpannicularly. Thirty-nine implanted subpannicularly with 20-mg. pellets.

†This female carried stilbestrol pellets for 345 days starting on the 47th day of life. There were minute tumor nodules in each kidney; no luteinization was detected in either ovary.

TABLE 4.—Occurrence of renal tumors in untreated and in estrogen-treated ovariectomized hamsters

	Number in sample	Mean age when ovariectomized, with range (*days*)	Mean duration after ovariectomy, with range (*days*)	Estrogen used	Mode of administration	Mean age at start of estrogen treatment, with range (*days*)	Mean duration of estrogen treatment, with range (*days*)	Mean age at autopsy, with range (*days*)	Renal tumors	Metastases
Untreated, ovariectomized **hamsters**	12	31 30–47	412 317–423	—	—	—	—	443 347–453	0	0
Estrogen-treated ovariectomized hamsters	7	39 30–52	211 163–267	Stilbestrol	One pellet	42 30–52	208 163–246	250 149–297	5 or 71%	0
	5	50 50–50	303 222–411	Stilbestrol	Injection + one pellet*	51 51–51	302 221–410	352 271–410	5 or 100%	0
	4	174 66–101	201 196–255	Stilbestrol	One pellet + one ovary in spleen†	124 98–132	204 164–224	327 262–356	2 or 50%	0
	9	32 31–33	209 150–296	Stilbestrol	Five pellets	50 49–51	191 131–277	241 182–327	5 or 56%	1
	16	47 26–119	222 121–354	Estradiol	One pellet	55 50–119	214 121–354	269 171–404	10 or 63%	0
Total: estrogen-treated ovariectomized hamsters	**25**	47 30–101	232 150–411	Stilbestrol		60 30–132	220 131–410	280 149–410	17 or 68%	1
	41	45 26–119	218 121–411	Stilbestrol + estradiol		55 30–132	208 121–410	263 149–410	27 or 66%	1

*Subpannicular injection of 0.6 mg. every 2d day, in sesame-oil or in saline suspension, followed by 20-mg. pellets, subpannicularly.

†None of the splenic ovarian grafts showed growth.

Table 5.—Incidence of renal tumors in intact female hamsters in which estrogen treatment* was started between the middle of metestrum B and the middle of proestrum

	Number of animals in sample	Age when treatment started, with range	Duration of treatment, with range	Mean age at autopsy, with range	Renal tumors		Metastases
					Left	Right	
Late metestrum B, 8 a.m. of day 1	3	95 94–95	219 190–242	314 284–337	0	0	0
Middle proestrum, 4:30 p.m. of day 1	5	96 95–98	236 214–288	332 309–383	0	0	0
Estrum, 8 p.m. of day 2	18	109 107–113	256 163–366	365 273–474	0	0	0
Middle metestrum B, 7:30 p.m. of day 4	5	95 95–95	299 253–349	394 348–444	0	1	0

*Subpannicular injection of 0.6 mg. estradiol dipropionate in 0.12-cc. sesame-oil solution and 20-mg. pellet stilbestrol implanted subpannicularly.

TABLE 6.—Absence of renal tumors in intact male, in orchiectomized, and in ovariectomized hamsters treated with stilbestrol and progesterone administered together as subpannicularly implanted 20-mg. pellets

	Number in sample	Mean age when gonadectomized, with range	Mean duration of treatment, with range (*days*)	Mean age at autopsy, with range (*days*)	Renal tumors
Intact males	11	—	361 293–452	397 343–416	0
Orchiectomized	14	55 50–63	399 399–400	454 450–462	0
Ovariectomized	13	58 57–59	287 219–401	345 278–460	0

TABLE 7.—Absence of renal tumors in hamsters treated with progesterone administered as subpannicularly implanted 20-mg. pellets

	Number in sample	Mean age when gonadectomized, with range	Mean age at start of progesterone treatment, with range	Mean duration of progesterone treatment, with range	Mean age at autopsy, with range	Renal tumors
Intact males	15	—	50 50–50	415 413–418	465 463–468	0
Intact females	15	—	50 50–50	359 238–414	442 288–464	0
Orchiectomized	15	47 46–50	50 50–50	410 370–414	460 420–464	0
Ovariectomized	14	48 47–48	50 50–50	404 268–415	454 318–465	0

injected daily with ACTH. Although the period of survival was short for all 4 animals, 1 acquired tumors in each kidney (table 11).

The observations described are summarized in tables 12 and 13.

In our laboratory the use of estrogen-treated female hamsters has been somewhat handicapped by the susceptibility of the ovarian bursae and uterine horns of such animals to a small, unidentified, gram-negative, abscess-forming bacillus. Abscesses containing immense numbers of these organisms are very common and frequently attain relatively huge size (figs. 13 and 14). The growth of the organism is markedly inhibited by chloromycetin and streptomycin, slightly inhibited by aureomycin, and not inhibited at all by penicillin. Less frequently, the same organism is found in prostatic abscesses, especially in males receiving stilbestrol treatment from the first 2 or 3 days of life (fig. 16).

DISCUSSION

In intact male or in gonadectomized Syrian hamsters of either sex, the induction of malignant renal tumors by treatment with estrogen (tables 1, 2, and 4) is known to be inhibited by either progesterone, testosterone

TABLE 8.—Incidence of renal tumors in intact female hamsters in which estrogen treatment was started during periods of estimated low progesterone secretion*

	Number of animals in sample	Estrogen	Androgen	Mean age when estrogen treatment started, with range	Mean duration of estrogen treatment, with range	Estimated maximum duration of androgen treatment (*days*)	Mean age at autopsy, with range (*days*)	Renal tumors	Metastases
Estrogen on day 1 or 2 of life	4	20-mg. pellet stilbestrol	—	2 1–2	201 181–232	—	202 183–232	3 or 75%	0
Estrogen on day 10 of life	5	20-mg. pellet stilbestrol	—	10 10–10	373 345–400	—	383 355–411	3 or 60%	0
Estrogen on day 18 of life	8	20-mg. pellet stilbestrol	—	18 18–18	401 344–426	—	419 362–444	4 or 50%	0
Androgen on day 1 of life; followed later by stilbestrol	20	20-mg. pellet stilbestrol	Test. prop. pellet†[a]	65 50–196	225 147–286	50	290 262–343	20 or 100%	1 or 5% of 20
	1	20-mg. pellet stilbestrol	Test. prop. solution†[b]	50	246	30	296	1 or 100%	0
	2	20-mg. pellet	Test. phenyl acetate†[c]	50 50–50	247 247–247	25	297 297–297	2 or 100%	0
Androgen on day 1 of life; no subsequent treatment	10	—	Test. prop. pellet†[a]	—	—	50	372 270–433	0	0

*For data on renal tumors in animals with estrogen treatment started during middle metestrum B *see* table 5.

† [a] Testosterone propionate pellets; about 4 mg.; subpannicularly, no longer palpable after 50 days or less.

[b] Testosterone propionate solution; 1.0 mg. in 0.1 cc. sesame oil; intraperitoneally.

[c] Testosterone phenyl acetate, aqueous suspension; 2.5 mg. in 0.5 cc.; intraperitoneally.

TABLE 9.—Incidence of tumors in portions of kidneys from newborn male and female hamsters transplanted subpannicularly in stilbestrol-treated male hosts on 51st day of life

Sex of donor	Accession No. of host	Duration of treatment (*transplant and stilbestrol*)	Age at autopsy (*days*)	Transplant remnant	Tumor found in transplant	Primary renal tumors		Metatases from primary tumors
						Left	Right	
♂	3177	213	264	—	—	—	+	—
	3433	331	382	+	+	+	+	—
	3467	350	401	+	+	+	+	—
	3472	352	403	+	+	+	+	+
	3522	374	425	+	+	+	+	—
♀	3100	212	263	—	—	—	—	—
	3101	212	263	+	—	—	—	—
	3364	288	339	—	—	+	+	—
	3366	289	340	—	—	—	+	—
	3372	294	345	+	+	+	+	—
	3413	318	369	+	+	+	+	—

TABLE 10.—Incidence of renal tumors in adrenalectomized, male hamsters treated with stilbestrol and cortisone administered as subpannicularly implanted 20-mg. pellets

Number of animals in sample	Mean age when pellet implanted, with range	Mean age when adrenalectomized, with range	Mean duration of treatment with stilbestrol and cortisone, with range	Mean duration of adrenalectomy, with range	Mean age at autopsy, with range	Renal tumors
4	51 51–51	53 53–53	154 147–158	152 145–156	205 198–209	3

TABLE 11.—Incidence of renal tumors in male hamsters implanted subpannicularly with pellets of stilbestrol and injected daily, subpannicularly, with 0.5 cc. or 0.75 units of ACTH

Accession No.	Preparation of ACTH used	Age when treatment started (*days*)	Duration of treatment (*days*)	Renal tumors	
				Left	Right
2215	Armour	51	175	—	—
2356		51	252	—	—
2166	Rinfret	58	121	—	—
2287		58	201	+	+

propionate, or deoxycorticosterone acetate (*4*). The chronic treatment of male Syrian hamsters with estrogen is followed by marked involution of both testes and accessories (*27*). Consequently, it may be assumed that the intact male, under such treatment, fails to secrete sufficient androgen to prevent tumor induction. That similar treatment of mature, intact females ordinarily fails to result in renal-tumor formation suggests the

TABLE 12.—Summary of relation between estrogens and progesterone and the incidence of estrogen-induced renal tumors in intact and gonadectomized male and female hamsters

Treatment*	Sex	Number of animals in sample	Renal tumors
None	♂	61	0
	♂̸	60	0
	♀̸	12	0
	♀	84	0
Stilbestrol	♂	213	177 or 83%
Estradiol	♂	15	15 or 100%
Estrone	♂	8	7 or 88%
Stilbestrol	♂̸	61	47 or 77%
Estradiol	♂̸	12	12 or 100%
Estrone	♂̸	10	10 or 100%
Stilbestrol	♀̸	21	16 or 76%
Estradiol	♀̸	16	10 or 63%
Stilbestrol	♀	43	1 or 2%
Estradiol	♀	6	0
Estrone	♀	1	0
Stilbestrol and progesterone	♂	11	0
	♂̸	14	0
	♀̸	13	0
	♀	15	0

*All treated animals received treatment for 250 days or longer and were autopsied at 300 days of age or older; consequently data do not necessarily match exactly those given in tables 1, 3, 4, and 13.

TABLE 13.—Summary of 5 methods of inducing tumors in the kidneys of female hamsters

Treatment*	Number of animals in sample	Renal tumors
Ovariectomy plus estrogen	41	27 or 66%
Estrogen treatment started during middle metestrum B	5	1 or 20%
Estrogen treatment started on 2d day of life	3	2 or 67%
Estrogen treatment started on 10th day of life	5	3 or 60%
Estrogen treatment started on 18th day of life	8	4 or 50%
Testosterone propionate on 1st day of life; replaced by stilbestrol on 50th or 196th day of life	20	20 or 100%
Kidney from newborn female donor transplanted subpannicularly in stilbestrol-treated male host	6	2 or 33%

*Estrogens: stilbestrol or estradiol.

possibility that the ovaries of such animals may secrete sufficient progesterone to prevent tumor induction.

That antagonists of adrenal origin may be responsible for the failure is rendered unlikely by the fact that renal tumors may be induced readily in the adrenalectomized, stilbestrol-treated, male hamster maintained with the aid of cortisone (table 10). The critical experiment with females has not yet been performed. It must be remembered that there are good reasons for suspecting the frequent occurrence of accessory adrenals in the Syrian hamster (*28*), although, as far as I am aware, none have yet been recorded. In our colony only one accessory adrenal gland has been observed; this was found at autopsy, just external to the capsule of the right adrenal gland in a 243-day-old female treated for 169 days with stilbestrol and testosterone propionate and an intrathoracically transplanted flank organ tumor. It is planned to consider this problem more fully in a subsequent paper.

Pregnanediol determinations of estrogen-treated female hamsters have not been reported, but preliminary histological examinations of ovaries from such animals have indicated the presence of luteinization (fig. 15). This suggestion is in harmony with reported observations, in rats, rabbits, and guinea pigs, that the administration of exogenous estrogen enhances any existing L.H. (luteinizing hormone) output, *i.e.*, when corpora are present they enlarge and remain functional, as during estrum, but when they are absent, as during diestrum or in immature females, the ovaries become smaller and no corpora are formed (*29*). In the mature female hamster the time of lowest progesterone secretion should be between the middle of metestrum B, as defined by Ward (*22*), and the middle of proestrum, during which the corpora are almost completely regressed (*30*) and probably insensitive to L.H. stimulation [which the pituitary gland is probably still secreting (*31*)], but follicles destined to ovulate during the next estrum may or may not be sensitive. Probably the best time for starting estrogen treatment is late in metestrum B, or at about 8 a.m. of day 1 of the cycle, since L.H. sensitive corpora are least likely to be present then. Our own data (table 8) are consistent with this concept, since with one exception, the only renal tumor found among the mature, intact females was in one animal that received estrogen during middle metestrum B, *i.e.*, at 7:30 p.m. of day 4 of the cycle. The single exception was the stilbestrol-treated female listed in table 3 as having minute tumor nodules in each kidney when autopsied after 345 days of treatment; the stage of the cycle in which treatment was started in this animal is not known but the ovaries showed no luteinization when examined microscopically. The occasional occurrence of renal tumors is to be expected in female hamsters in which chronic treatment with estrogen was started at unknown times during the estrous cycle. Among an unreported number of females treated with stilbestrol for 8 months, a single renal tumor was reported by Paschkis *et al.* (*21*).

The late metestrum B group, injected with estradiol and implanted with stilbestrol at 8 a.m. of day 1, consisted of only 3 animals surviving 109 days of treatment; none of these 3 survived beyond 242 days of treatment. It is likely that increasing the size of this group and extending the duration of treatment to between 250 and 300 days would permit the demonstration of renal tumors. Timing is extremely important here, since for tumor induction, an adequately low concentration of progesterone in the blood is probably of very short duration.

It is very interesting that Horning (*18*) observed renal tumors in two pregnant hamsters treated with stilbestrol, though he was unable to induce the tumors in intact virgins. He then attempted to induce tumors in the kidneys of females by administering stilbestrol and progesterone combined. In view of the observations reported here it is not surprising that the experiment was unsuccessful. None of my own females, injected 7 times, at 48-hour intervals, with 0.10, 0.05, or 0.01 mg. of stilbestrol in sesame-oil solution, starting on the 5th day of pregnancy, delivered litters. Since the treatment was not continued no renal tumors were expected and none were found.

The occurrence of renal tumors in immature, intact females treated with estrogen is interpreted as indicating the failure of the pituitary gland of such immature animals to respond to estrogen by L.H. secretion, rather than to a failure of the immature ovaries to respond to L.H. stimulation. This interpretation may be an oversimplification, however, since data summarized in table 8 suggest that the incidence of induced renal tumors decreases with the advancing prepuberal age (from 1 to 18 days) at the time estrogen treatment is started. The data suggest, also, that there may be a progressive lengthening of the latent period for tumor induction over this 18-day period. Presumably there is no significant increase in the secretion of progesterone, or of progesterone-like substances, during the prepuberal period.

As early as 1935 for the rat (*23*, *24*) and 1939 for the mouse (*25*), it was reported that the anterior hypophysis is bipotential at birth and capable of being irreversibly differentiated as either male or female in type, depending on whether a testis or an ovary is present. In the "masculinized" female little or no L.H. is released, constant estrus results, and the pituitary gland undergoes enlargement. It was concluded that any of the effects produced by the injection of large amounts of estrogen could be brought about by endogenous hormone alone provided an adequate endocrine imbalance were established (*25*).

The newborn female hamster might respond to androgen as the rat and mouse do (*32*) since corpora lutea are not formed in mature female hamsters treated earlier with testosterone. Assuming this similarity in response to androgen to be true, and assuming that the failure of most intact, mature, estrogen-treated female hamsters to acquire renal tumors is due to progesterone, produced during L.H. stimulation after the estrogen administration, it should be possible to induce the tumors in such "masculinized" female hamsters. In fact, since "masculinized" female rats and mice remain in estrus, exogenous estrogen might prove unnecessary, *i.e.*, the constantly secreted endogenous estrogen might be adequately tumorigenic.

Tumors may be induced in the "masculinized" female hamster by exogenous estrogen, which is demonstrated clearly by data in table 8. Other data in the same table make it equally clear that the endogenous estrogen secreted by these animals has not induced renal tumors. This failure is presumably owing to a level of secreted estrogen that is too low, *i.e.*, less than 0.03 mg. per day (*7*).

That there is no fundamental difference between the kidneys of male and female hamsters with respect to their susceptibility to the tumorigenic properties of estrogen is demonstrated also (table 9) by the development of typical renal-tumor tissue in kidney fragments transplanted subpannicularly in stilbestrol-treated male hosts, from newborn female and male donors (figs. 11 and 12).

While an adrenal involvement in the induction of renal tumors has not been established, adrenal ascorbic acid studies have shown that stilbestrol treatment in hamsters is associated with a release of more ACTH in females than in males (*33*), or gonadectomized males and females (*34*). This suggests the possibility that excess of ACTH in the stilbestrol-treated female may inhibit renal-tumor induction indirectly by stimulating the secretion of tumor-inhibiting steroids in the adrenal glands. Preliminary data presented in table 11 render the likelihood of such an inhibiting action improbable, since one male receiving both stilbestrol and ACTH for 201 days showed small tumor nodules in each kidney at autopsy (fig. 17).

The possibility has been suggested (*8*, *35*) that the failure of the female hamster to acquire renal tumors under estrogen treatment may be attributable to some degree of physiological adaptation to estrogen due to exposure of the female to plentiful amounts of this hormone during life. It is obvious that any such adaptation must disappear after ovariectomy and that it may be overcome by initiating estrogen treatment at any time, which is characterized by relatively low progesterone secretion.

If the role of exogenous progesterone as an inhibitor of renal-tumor induction in the estrogen-treated female hamster is as suggested in this paper, it should be possible to overcome the inhibition by increasing considerably the amount of estrogen administered, *e.g.*, by implanting 5, instead of one, 20-mg. pellets of stilbestrol. This experiment has been started and its results will be reported at a later time.

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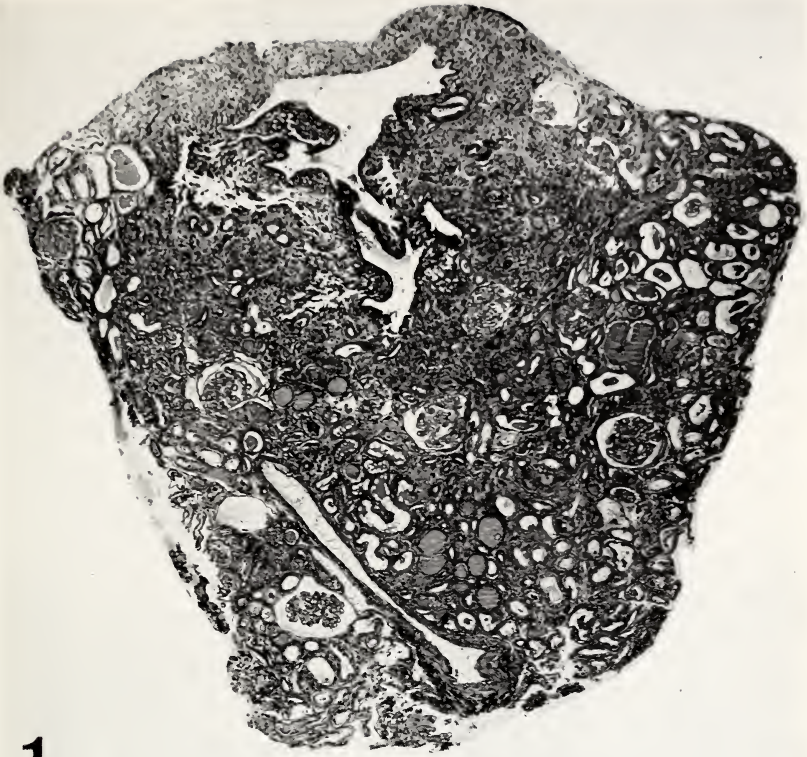
PLATE 11

FIGURE 1.—One of 2 small tumor nodules observed at autopsy in the left kidney of 1 of 49 intact female hamsters, implanted subpannicularly with one 20-mg. pellet of stilbestrol; reimplanted with stilbestrol after 150 days; total duration of treatment 345 days starting on the 47th day of life. × 45

FIGURE 2.—Tumors in right kidney of 1 of 9 ovariectomized hamsters, implanted subpannicularly with five 20-mg. pellets of stilbestrol; reimplanted with stilbestrol after 150 days; total duration of treatment 277 days starting on the 50th day of life. × 8



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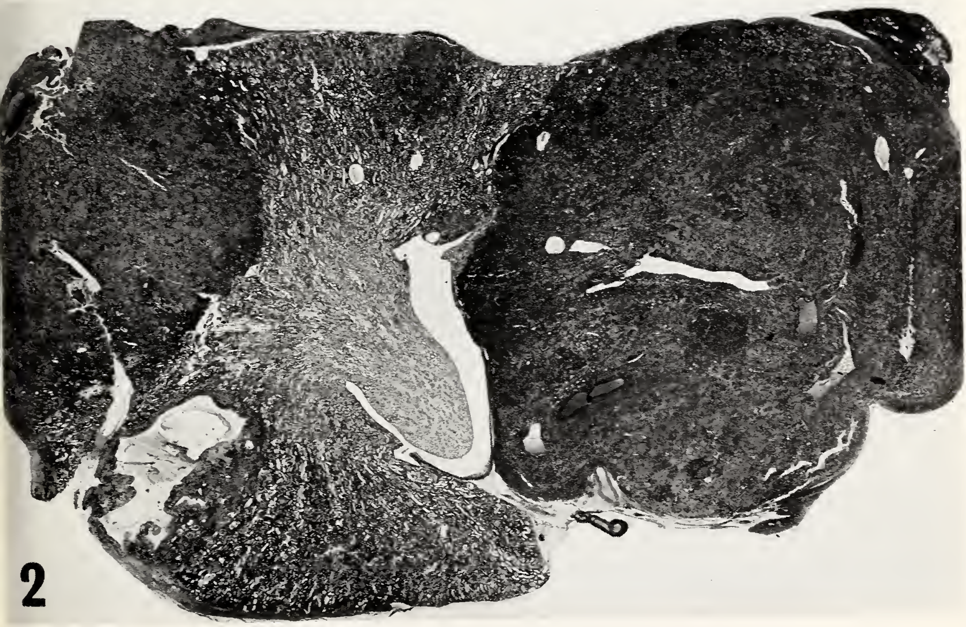

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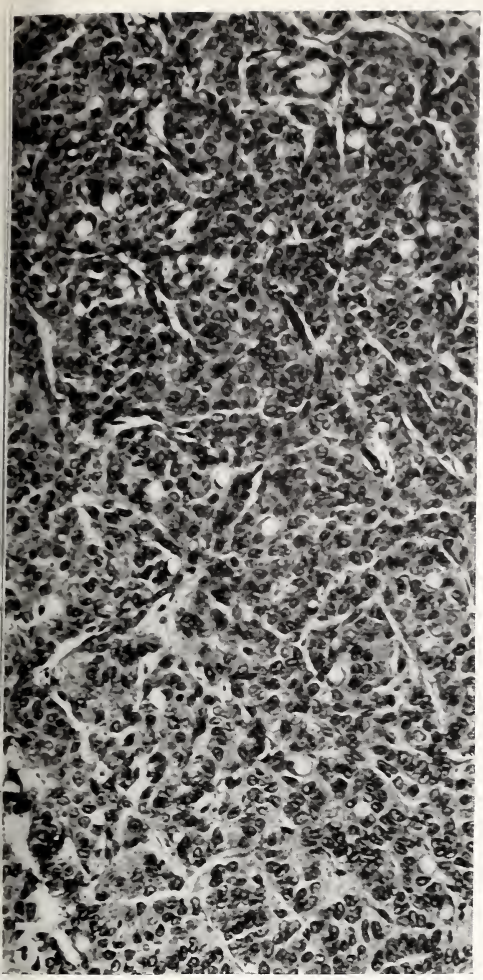
Plate 12

Figure 3.—One of the abundant, abdominal metastases derived from one of the primary renal tumors illustrated in figure 2. × 160

Figure 4.—Tumors in left (A) and right (B) kidney of an ovariectomized hamster, implanted subpannicularly with one 20-mg. pellet of estradiol for 294 days, starting on the 51st day of life. × 40

Figure 5.—Tumors in right kidney of a hamster receiving 0.6 mg. of estradiol in 0.12 cc. of sesame oil and a 21-mg. pellet of pure stilbestrol, subpannicularly, at 7:30 p.m. of day 4 of the cycle, *i.e.*, during the middle of metestrum; reimplanted with stilbestrol after 150 days; total length of treatment was 253 days. × 24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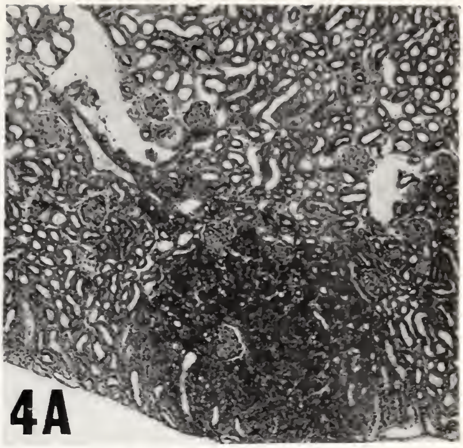
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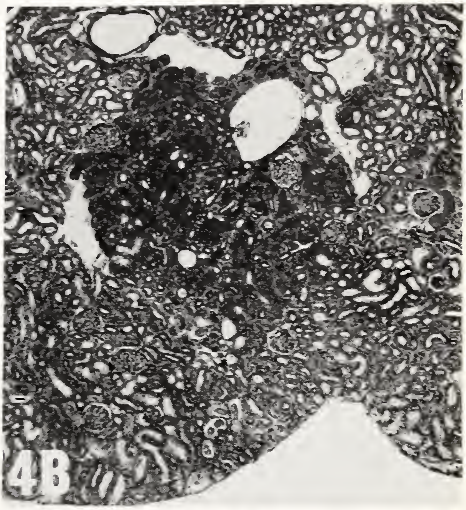
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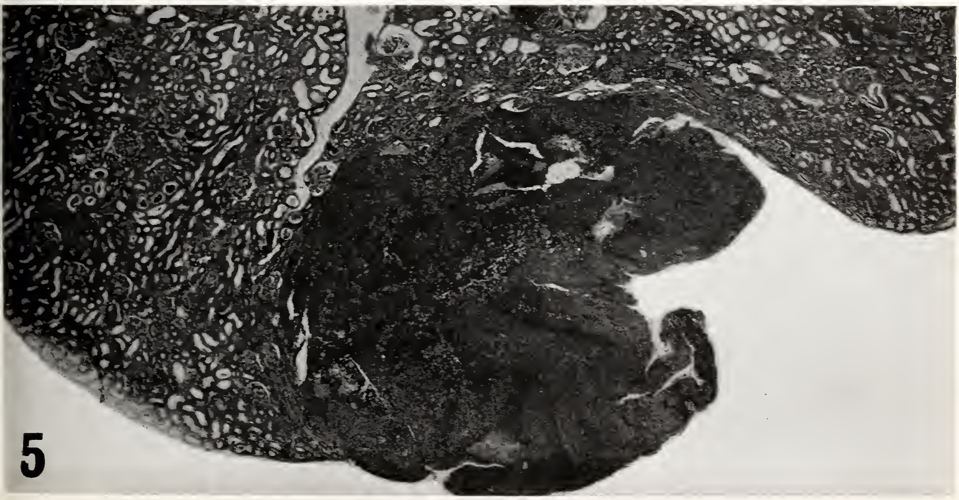
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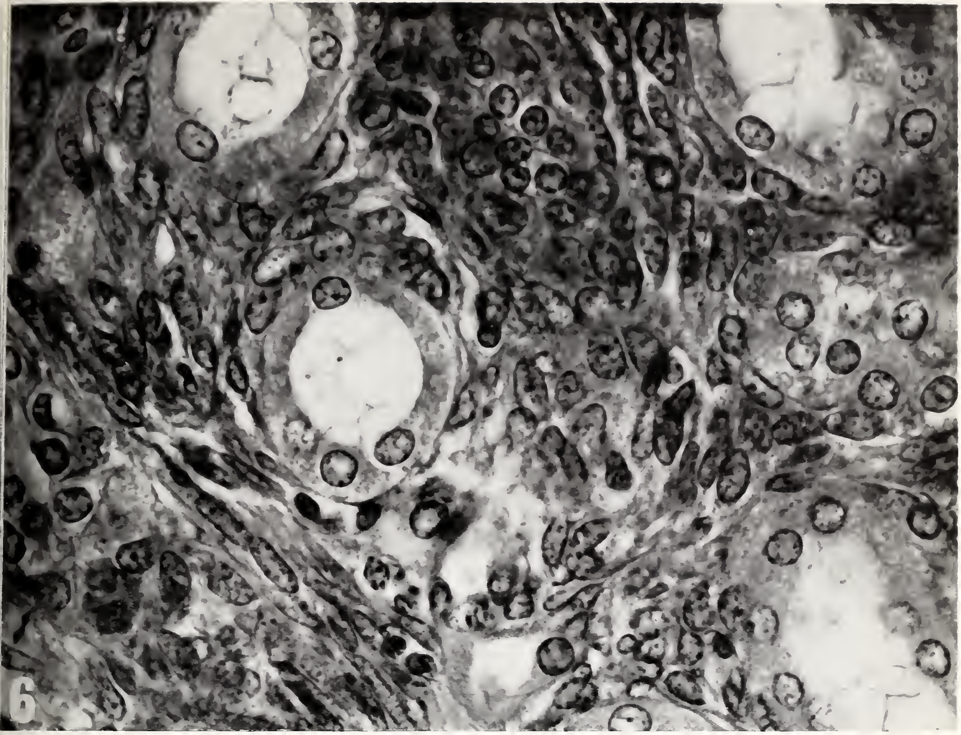
Plate 13

Figure 6.—Tumor in right kidney of 1 of 5 female hamsters, implanted subpannicularly with one 20-mg. pellet of stilbestrol on the 10th day of life; reimplanted with stilbestrol after 150 days; treatment continued for 400 days. × 540

Figure 7A.—Papillary tumor in right kidney of female hamster, implanted subpannicularly with one 20-mg. pellet of stilbestrol on the 18th day of life; reimplanted with stilbestrol after 150 days; total duration of treatment 426 days. × 40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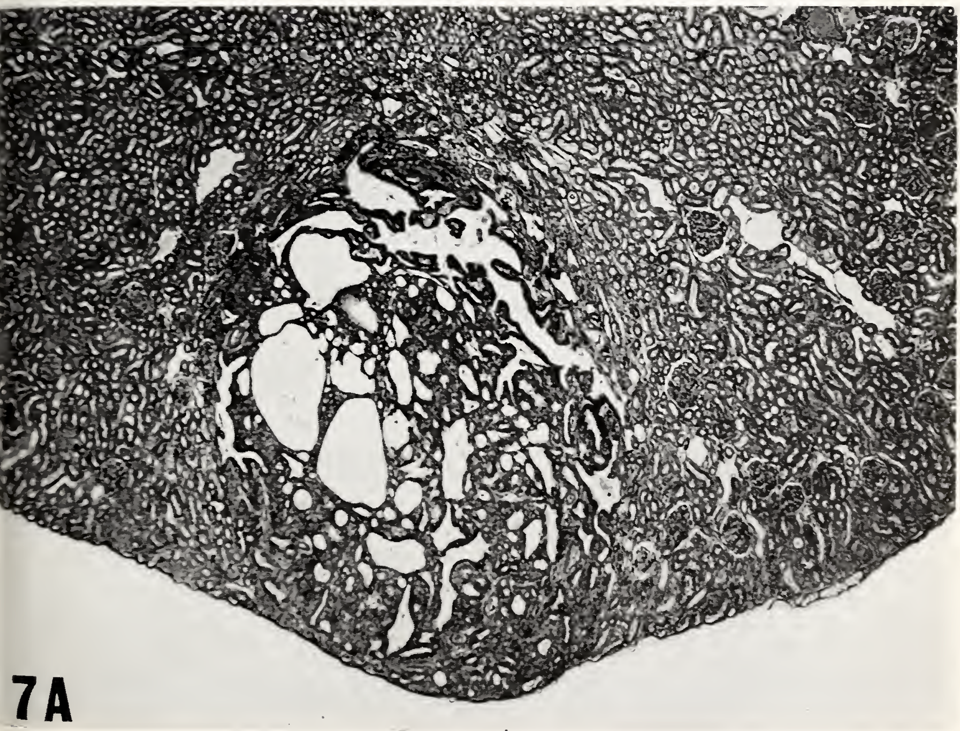
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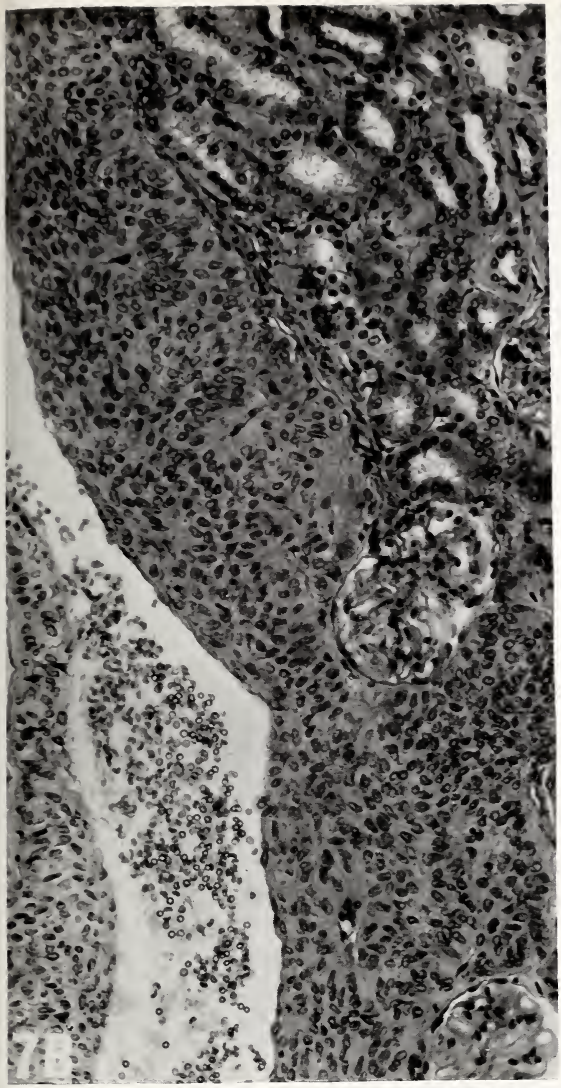
Plate 14

Figure 7B.—Tumor in left kidney of female hamster; treated as for 7A, but a different animal. × 160

Figure 8.—Tumor-containing, right and left kidneys from 1 of 20 female hamsters, implanted subpannicularly with one 2–5-mg. pellet of testosterone propionate on the day of birth and with one 20-mg. pellet of stilbestrol on the 50th day of life; reimplanted with stilbestrol after 150 days; treatment with stilbestrol continued for 286 days. [Reprinted by permission of *Cancer, see* (*19*).] × 1

Figure 9.—One of the abundant, abdominal metastases derived from the primary renal tumors illustrated in figure 8. × 160



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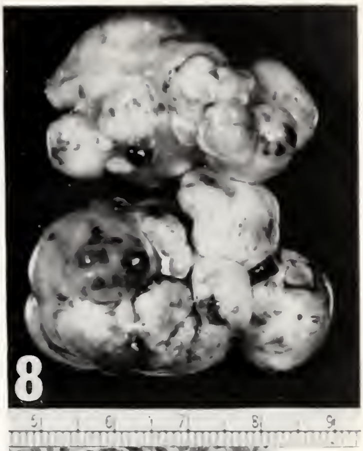

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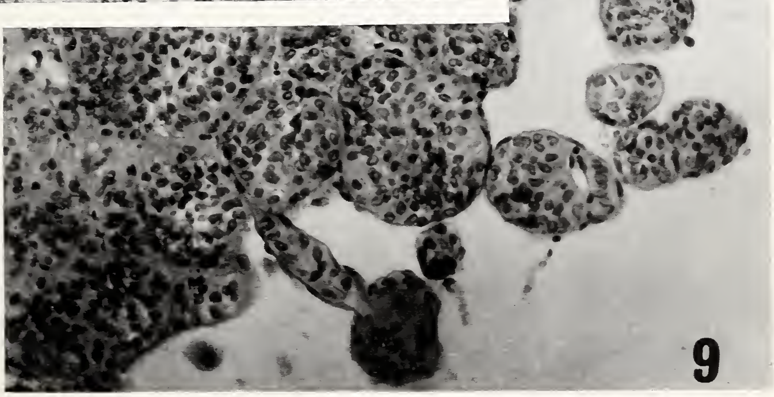

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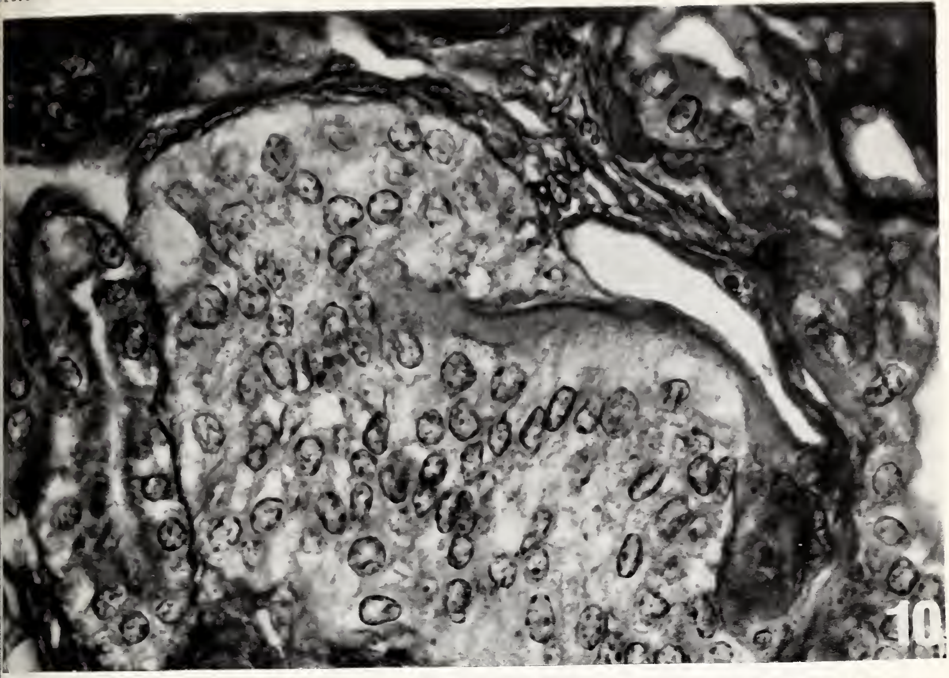
PLATE 15

FIGURE 10.—Tumor in right kidney of 1 of 20 female hamsters, implanted subpannicularly with one 2–5-mg. pellet of testosterone propionate on the day of birth and with one 20-mg. pellet of stilbestrol on the 50th day of life; reimplanted with stilbestrol after 150 days; treatment with stilbestrol continued for 211 days. Convoluted tubule near upper-right corner shows the characteristic increase in cell height preliminary to neoplastic change. × 540

FIGURE 11.—Tumor in cystic remnant of one-third kidney transplanted subpannicularly from a newborn female hamster to six 51-day-old male hamsters. Hosts were implanted subpannicularly with 20-mg. pellets of stilbestrol at time of transplantation; estrogen was reimplanted 150 days later; total duration of treatment was 294 days. × 720



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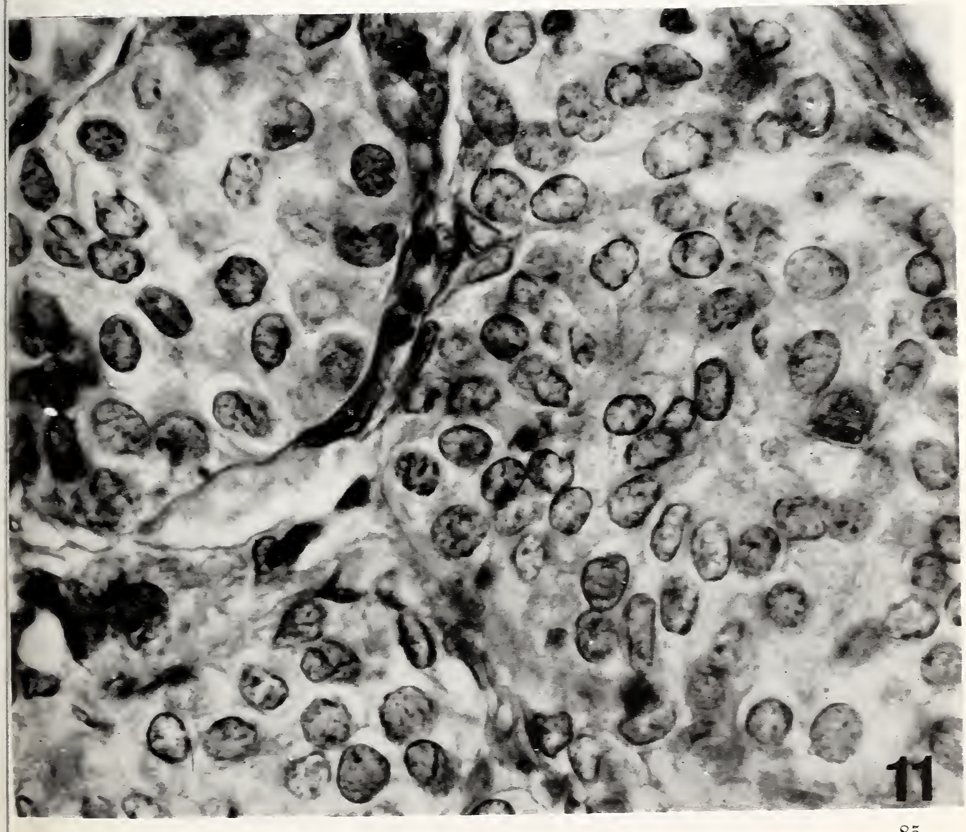

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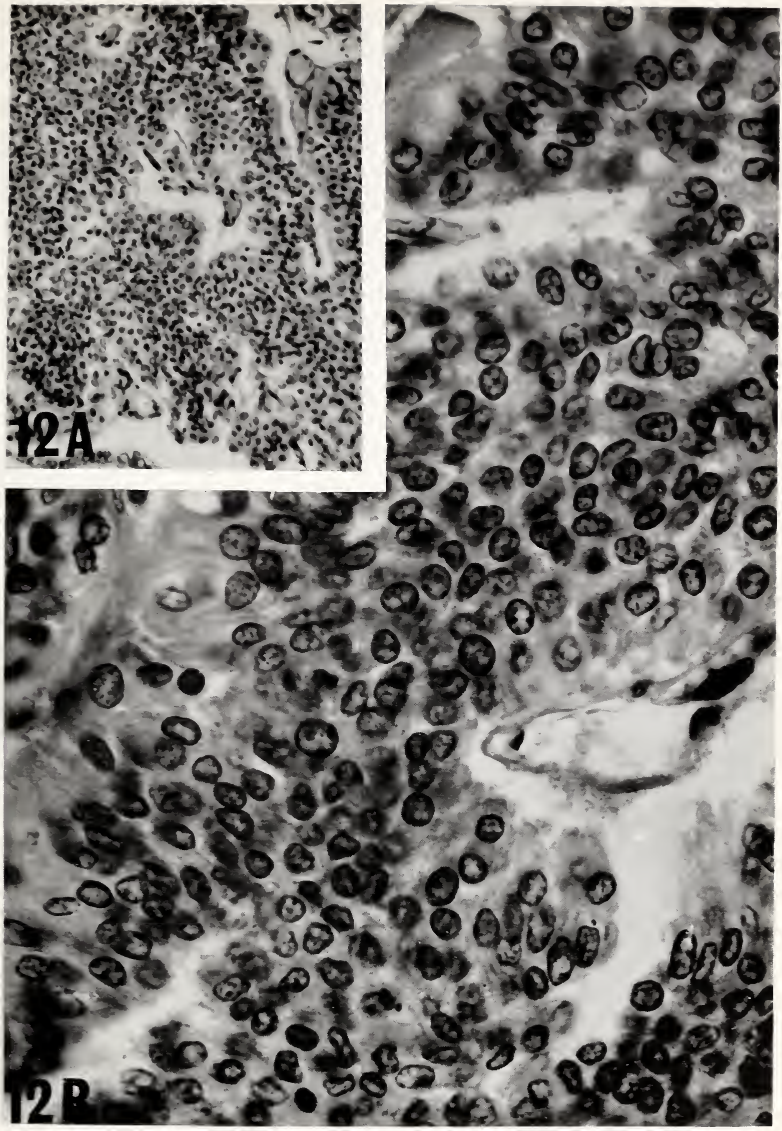
PLATE 16

FIGURE 12.—Tumor in cystic remnant of one-third kidney transplanted subpanniculary from a newborn male hamster to five 51-day-old male hamsters. The hosts otherwise were treated the same as the animal described in figure 11; total duration of treatment was 350 days. (A) × 160; (B) × 540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12B

Plate 17

Figure 13.—Dorsal aspect of a female hamster implanted subpannicularly with one 2–5-mg. pellet of testosterone propionate on the day of birth and with one 20-mg. pellet of stilbestrol on the 51st day of life; reimplanted with stilbestrol after 150 days; total duration of treatment with stilbestrol was 264 days. The marked abdominal distention was due to the uterine abscesses illustrated in figure 14. × 0.66

Figure 14.—The same animal illustrated in figure 13. The ventral body wall had been opened to expose the purulent uterine horns which contained 72 gm. of pus, without which the body weight of the animal was 90 gm. × 0.66

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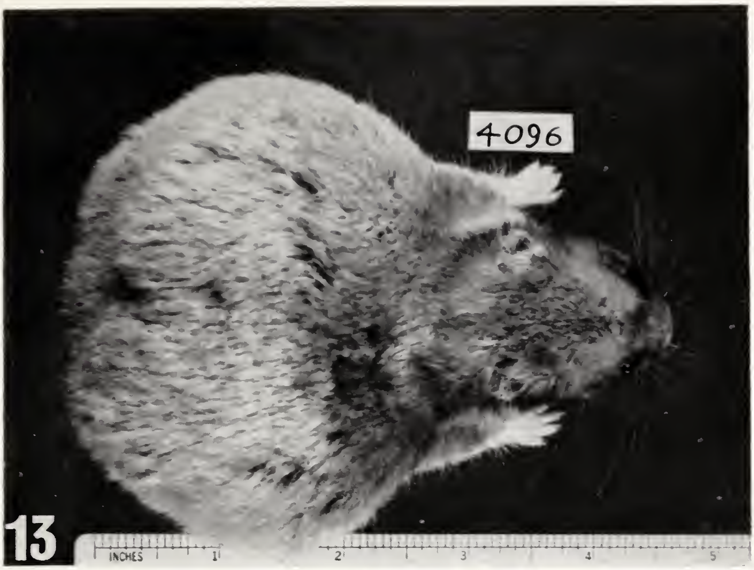
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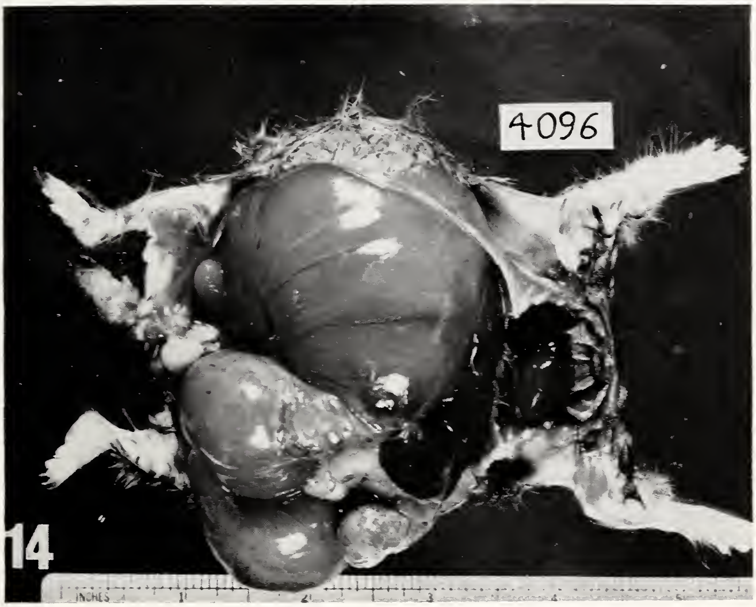
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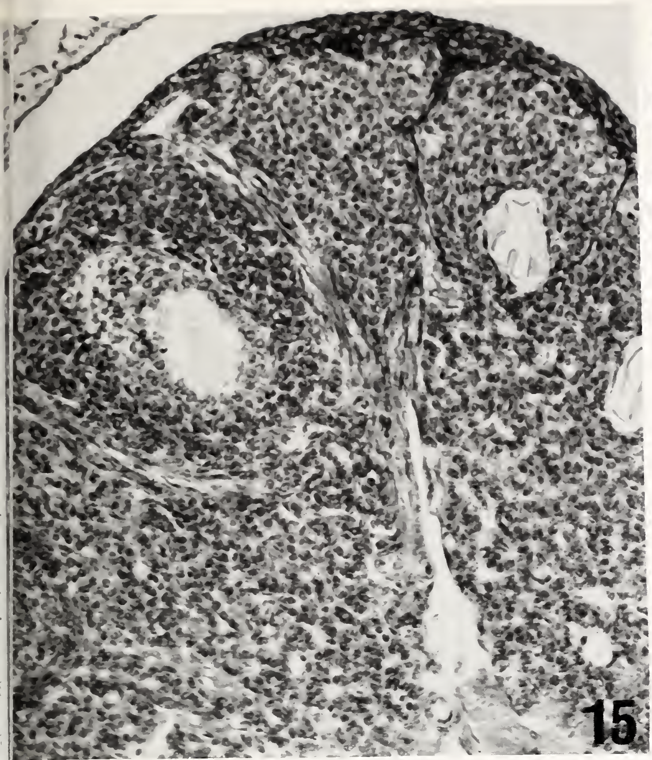
PLATE 18

FIGURE 15.—Luteinization in an ovary from a hamster receiving an injection subpannicularly, on the 43d day of life, of 0.6 mg. of stilbestrol in 0.4 cc. of sesame-oil solution; treatment was continued every 2d day for 107 days when the injections were changed to the same amount of stilbestrol in the form of microcrystals suspended in NaCl solution; this was continued for 214 days; total duration of treatment was 321 days. × 160

FIGURE 16.—Prostatic abscess involving seminal vesicles in male hamster, implanted with one 5-mg. pellet of stilbestrol on the 2d day of life and reimplanted with 20-mg. pellets of stilbestrol 27 and 209 days thereafter; total length of treatment was 245 days. × 0.66

FIGURE 17.—Tumors in right kidney of a male hamster, implanted with a 20-mg. pellet of stilbestrol on the 58th day of life; reimplanted with stilbestrol 150 days later. Also, on the 58th day of life the animal received an injection subpannicularly of 1 mg. of equine ACTH; these injections were continued daily until death of the animal 201 days later. Tiny tumors were observed in each kidney at autopsy. (A) × 480; (B) × 600



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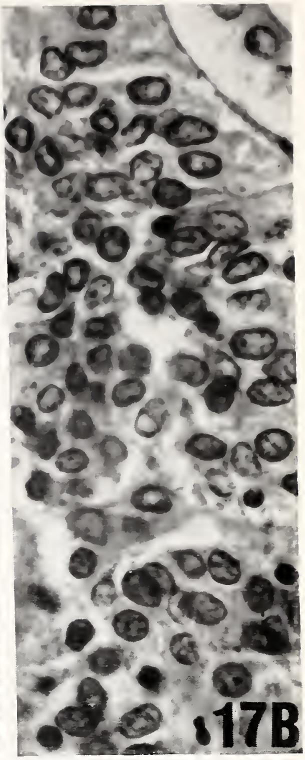

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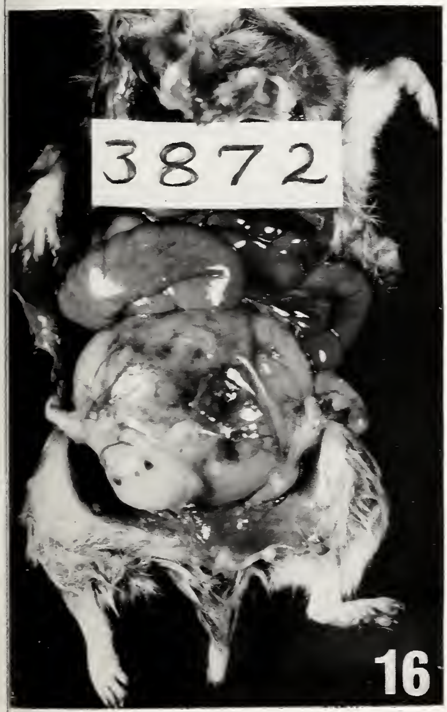

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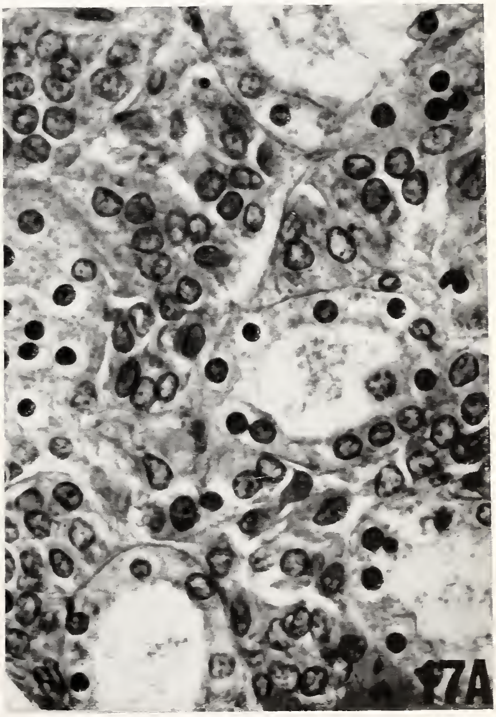

17A

Estrogen-Induced Tumors of the Kidney. V. Histology and Histogenesis in the Syrian Hamster [1, 2, 3]

HADLEY KIRKMAN *and* MARILYN ROBBINS,[4, 5] *Department of Anatomy, Stanford University School of Medicine, Stanford, California, and the Chester Beatty Research Institute, Institute of Cancer Research, Royal Cancer Hospital, London, England*

SUMMARY

The macroscopic and microscopic structure and histogenesis of the estrogen-induced renal carcinoma of the Syrian hamster are described. Macroscopically the tumor is bilateral and multifocal with a tendency to form cystic, often hemorrhagic, cavities during later stages of development. It is not encapsulated. Metastases are intra-abdominal at first, but reach the lungs and cervical lymph nodes later. Healthy tumor nodules are white, but turn amber during regression. Microscopically, tumor cells tend to be arranged in branching, anastomosing cords and plates of variable thickness. Cystic spaces are common; papillary formations are seen sometimes. Cells frequently form rosettes around thin-walled vessels. Most tumor cells are cuboidal; later they may become columnar or spindle-shaped. Hosts carrying primary tumors may survive for 500 days. Tumor cells have moderately hyperchromatic nuclei and lightly basophilic cytoplasm containing slender, rod-shaped, and filamentous mitochondria, medium-sized, eccentric Golgi nets, doubly refractive lipides, cholesterol and cholesterol esters, alkaline and acid phosphatase, and ascorbic acid; glycogen is absent. Ciliated cells are common; in many tumors two cell types occur—when present they do not mix indiscriminantly, but appear in segregated groups. Histogenesis of the tumor is not clear. It is certain that most cells arise from tubular epithelium, and the proximal convolution is definitely involved, but origins from distal segments are probable. It is believed that an intertubular contribution may occur also. In attempts to classify the tumor and to study its histogenesis, some skepticism is expressed concerning the adequacy of strictly morphological criteria.

FOLLOWING THE original interpretation of Horning and Whittick (*1*), it is generally assumed by investigators, familiar with the estrogen-

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[1] Received for publication December 29, 1958; revised May 25, 1959.

[2] This investigation was supported in part by research grants from the American Cancer Society, Inc., and the National Cancer Institute, National Institutes of Health, Public Health Service.

[3] The experimental work reported here was completed at Stanford University; the data were assembled and the manuscript was prepared during the senior author's tenure of a Senior National Science Foundation Fellowship (1957-58) and a Special U.S. Public Health Service Fellowship (1958-59) at the Chester Beatty Research Institute.

[4] *Present address:* Palo Alto Research Foundation, Palo Alto, Calif.

[5] The authors wish to thank Ciba Pharmaceutical Products, Inc., Summit, N.J., for supplying the diethylstilbestrol and various hormones used in the study, and the following individuals, for various technical services: Frank Barrett, Ray Crabbe, Gladys T. Kirkman, and Max Millsap, of Stanford University, and Michael J. Docherty, Robert McCulloch, Kenneth Moreman, and Edward A. Sykes, of the Chester Beatty Research Institute.

induced renal tumor of the hamster, that this neoplasm is exclusively of tubular origin and should be classified as a carcinoma.

In our laboratory we have never been certain as to the proper classification of the tumor in spite of its predominantly carcinomatous appearance. Our original report (*2*), based upon 6 diethylstilbestrol (stilbestrol)-treated males, referred to potentially malignant renal adenomas, and had little to say concerning their histogenesis. Two years later (*3*) we reported on renal adenomas and adenocarcinomas and suggested a tubular origin with a possible contribution from the epithelium of the renal pelvis. The following year (*4*) we adopted, tentatively, Ewing's term "malignant adenoma" and suggested a primary origin from the nephron, especially the proximal tubule, with possible contributions from the renal pelvis and from cells of the intertubular cortical stroma. In 1951 (*5*) we did not classify the tumor, but did state that "some tumor cells are now known to arise from interstitial connective tissue near the corticomedullary border, but epithelial origins are believed to occur also." Since then we have usually referred to the neoplasm simply as the estrogen-induced, malignant, renal tumor. However, in 1955 (*6*) we briefly reaffirmed our belief in a connective-tissue contribution and summarized our concept of the relative roles of tubular epithelium and stromal cells in the origin of the tumor nodules. The present paper presents, in much more detail than heretofore, our current ideas concerning the morphology and histogenesis of the tumor.

MATERIALS AND METHODS

The macroscopic observations reported are based chiefly on autopsy findings in 660 hamsters having primary renal tumors; occasional observations are included from autopsy findings in 624 hamsters transplanted with renal tumors; these 624 animals include 300 used in carrying the tumor through 32 serial passages. The microscopic observations are based on the study of preparations made from several hundred different renal tumors, including primary tumors and their metastases and transplants made from each. For routine histological study most material was fixed in Bouin's fluid and stained with hematoxylin and eosin or with "Mallory Azan." Other fixatives used for special purposes included: absolute methyl alcohol, acetic osmic bichromate, Carnoy's, Champy's, cold acetone, Dustin's, 80 percent ethyl alcohol, formol-acetic-alcohol, Kolatchew-Nassonow's, Lillie's buffered 10 percent formalin, Rossman's cold picro-formol-alcohol, SUSA, Zenker acetic, Zenker formol, and Zenker stock. Other stains used included: Sudan III, IV, and Sudan Black, Nile-blue sulfate, the Schultz reaction, digitonin with and without Sudan IV, Ashbel-Seligman carbonyl reaction, Gomori's acid and alkaline-phosphatase and lipase reactions, Thomas's arginine reaction, periodic acid-Schiff, Best's carmine, Bauer-Feulgen, mucicarmine, Congo red, methyl violet, safranin, alkaline thionine, $AgNO_3$ reaction for ascorbic acid, Verhoeff's elastic fiber stain, Foot's silver ammonium carbonate stain for reticular fibers, phosphotungstic acid-hematoxylin, Heidenhain's iron-alum-hematoxylin, Wright's stain, eosin methylene blue, aniline acid fuchsin with aniline blue counterstain, and intravital trypan blue. Other methods used were essentially as described in other papers (*7–10*).

OBSERVATIONS

Macroscopic Characteristics

After about 200 days of treatment, early stages of primary tumors may be seen on the surface of each kidney as slightly elevated, rounded, white, solid, subcapsular nodules. When such nodules are visible, and often when they are not, similar ones may be found at deeper levels, after freehand sectioning of the organ. Although the nodules are always cortically located, and may arise at any cortical level, their incidence tends to be somewhat greater near the corticomedullary border, adjacent to the arcuate vessels (fig. 1).

Later, *e.g.*, after 250 to 300 days of treatment, the number of nodules has increased and there may be a marked variation in nodule size, indicating a considerable range in the length of the latent period for induction of different nodules within the same kidney. Some of the larger nodules may be cystic and frequently hemorrhagic. As the central regions of larger tumor masses become necrotic their color changes from white to amber or creamy yellow.

After 300 days of treatment, and frequently earlier, each kidney usually has become greatly enlarged and presents an extremely distorted and irregular surface consisting of exposed, smoothly protruding portions of many solid or cystic, white, clear or hemorrhagic, tumor masses. Between adjacent masses remnants of apparently normal kidney tissue may be seen. The left kidney, normally slightly larger than the right, is usually the more involved, and sometimes attains a weight of 14 gm., or about 23 times the normal left-kidney weight of about 600 mg.

After 300 or more days of treatment metastases are likely to occur. At first these are entirely intra-abdominal, and arise partly by direct extension but mostly by the secondary implantation of small fragments released from primary lesions. It is not uncommon to find more or less necrotic, free-floating nodules within the peritoneal cavity (fig. 2). The most frequently encountered sites of secondary implantations are the peritoneal surface of the diaphragm, the splenic hilus, the omenta and mesenteries, the parietal peritoneum, the hepatic porta, and the scrotal sac. Invasion of the spleen occurs frequently; invasion of the liver less often. Secondary deposits upon the serosa of the liver or gastrointestinal tract are quite infrequent, but do occur. Occasionally, in long-treated animals, blood or lymph-borne metastases may be observed in the lungs and the cervical lymph nodes.

Microscopic Characteristics

In frozen sections of formalin-fixed material, stained with hematoxylin and Sudan, the earliest neoplastic lesions recognized as consisting of minute, intertubular clusters of few cells are distinctly sudanophilic, and contrast sharply in this respect with all surrounding healthy tubular epithelia (fig. 3). These sudanophilic areas are morphologically identical with similar areas found in serially sectioned, paraffin-embedded, hematoxylin and eosin-stained sections (fig. 4). When followed through serial sections, many of these tiny clumps of tumor cells fail to show any continuity or even intimate contact with adjacent tubular epithelium, *i.e.*, they appear to arise directly from some intertubular cellular element. Sudanophilia is demonstrable in the cells of atrophic tubules and within a few cells lining the renal pelvis near its attachment to the renal papilla. In autolyzed kidneys the nuclei of the small tumor foci do not undergo the rapid pyknosis characteristic of the nuclei of the tubular epithelium (fig. 5), which suggests a relative lack of catheptic enzymes in the tumor cells.

Tubular alterations consisting primarily of nuclear clumping, as observed normally in the macula densa of the distal tubule, increased cell height, and cytoplasmic basophilia may be fairly conspicuous (figs. 6, 7, and 8). Nuclear clumping is usually eccentric and is sometimes associated with amyloid deposition [fig. 10 (*11*)] and with variations in nuclear size (figs. 7 and 9); it is not always indicative of precancerous changes in the tubule (fig. 10) and is not confined entirely to estrogen-treated animals. An increase in the height of certain tubular cells, with augmented cytoplasmic basophilia and no nuclear clumping (fig. 7), is fairly common after relatively short periods of treatment and is considered as early neoplasia or preneoplasia, especially when the altered cells project beyond the normal base of the tubule, even though the basement membrane is still intact. These two alterations are not prevented by hormones, which tend to inhibit renal-tumor induction, *viz.*, progesterone, testosterone propionate, and deoxycorticosterone acetate (DCA). In fact, material so treated is sometimes especially favorable for detecting early stages in tubular neoplasia. Tumor cells arising under such conditions, however, are always in minute clumps representing abortive tumorigenesis (fig. 11). It is sometimes possible to indentify these tubular alterations in both proximal and distal tubules, and even in the parietal layer of the glomerular capsule (fig. 12).

All intrarenal collections of tumor cells, from the smallest to the largest, are clearly demonstrated, but never encapsulated. Their outlines are usually rather rounded, but may be quite irregular, since the masses expand by infiltration between tubules as well as by more uniform pressure against large areas of tubules. Expansion is associated with very little actual destruction of kidney tissue. Cords of tumor cells within the abdominal cavity, derived from primary foci by extension or by secondary implantation, are ordinarily covered by a thin layer of mesothelium (fig.

13). This layer is usually absent at the points of contact between the tumor and other normal tissue.

As a rule the tumor cells are arranged in the form of branching, anastomosing cords and plates of extremely variable thickness. The cords are enclosed by delicate networks of reticular fibers (fig. 14). Perithelial, rosette-like formations surrounding dilated, thin-walled vessels are quite common (fig. 15). On reaching the kidney surface, or the renal sinus, tumor cells tend to expand under the capsule (figs. 14 and 16).

Occasionally, especially after long periods of treatment, and in kidneys containing large tumors, there are extensive areas of long, spindle-shaped cells [figs. 6 (*8*), 17, and 18]. Most of these cells probably arise by metaplasia from cells of epithelial origin, although they tend to be enclosed by individual reticular fiber sheaths (fig. 19) and contain abundant cytoplasmic fibrils which do not appear to include true myofibrils. In a single instance, however, a small cyst appeared under the capsule of the right kidney of a male treated with stilbestrol for 374 days; the cyst was lined by a fairly thick layer of smooth muscle. The elongation of tumor cells appears to be particularly favored by administering either progesterone, DCA, or testosterone propionate with the stilbestrol.

Sometimes a very intimate relationship is demonstrable between tumor cells and reticular or collagenous fibers, *e.g.*, after "Mallory Azan" staining, the blue fibers appear to be in direct continuity with the red cytoplasm with no apparent cell membrane separating them. A similar relationship has been observed in the adrenal cortices of two 352-day-old male hamsters castrated on the 53d day of life.

The tumor-cell nuclei are slightly hyperchromatic and usually contain a single nucleolus of only moderate size. A preliminary count of 1,000 cells in a single tumor included only 5 mitotic figures. Mitoses do not appear to be particularly abnormal, except for the frequent abundance of chromosomal bridges (fig. 20); in the only estimate made, 111 of these were observed, within 30 minutes, under a 2-mm. oil-immersion objective and 8× oculars. The majority of tumor cells have but one nucleus; however, multinucleated cells do occur.

The lightly basophilic cytoplasm usually contains several small, sudanophilic, singly and doubly refractive droplets; occasionally these fuse to transform the cell into a small signet-ring type [figs. 6 (*7*) and 21]. When cysts occur within tumors, the clear fluid content, stained as an air-dried film, is not sudanophilic and contains no cholesterol. The cells give a positive Schultz test, but the digitonin reaction discloses only a few birefringent crystals and rosettes unstainable with Sudan III, *i.e.*, while some free cholesterol is present, chiefly at the periphery of the tumor masses where the healthiest cells are found, most cholesterol is in the form of cholesterides. No cholesterol has been observed among the tubule cells, though macrophages may give positive reactions with the Schultz test. The tumors give negative responses to the plasmal reaction and to the Ashbel-Seligman test for active carbonyl groups. They contain considerable alkaline phosphatase (figs. 22 and 23) and somewhat less demonstrable acid phosphatase. They stain well with the periodic acid-Schiff reaction but, unlike human renal carcinoma cells, contain no histochemically demonstrable glycogen. Thomas's test for arginine shows a slightly greater concentration of this amino acid in the tumor cells than in the tubular cells. Mucous cells occur, but are infrequent; they are common within a limited area of the pelvic epithelium of each sex. Nuclear, type B, inclusion bodies, resembling those found normally in the vasa deferentia of sexually mature mammals (*12*), may be observed but they are rare; much more common, especially in animals receiving either progesterone, testosterone propionate, or DCA with stilbestrol are globular, acidophilic, cytoplasmic inclusions (fig. 24), similar to those of young Russell body cells (*13*). Russell, who named them "fuchsine bodies," believed them to belong to the sprouting fungi and to cause anatomical changes in cancer (*14*). Recent work suggests that they are involved in antibody formation (*15*). Similar, but smaller, droplets occur occasionally in tubular cells near the corticomedullary junction; they stain deep red with azocarmine and light pink with eosin. Both the nuclear and cytoplasmic globules have been observed elsewhere in animals receiving similar or different treatment. The cytoplasmic globules are not the same as those characteristic of the globule leukocytes found in the urinary tract epithelium of the rat and elsewhere in certain other animals (*13*); they are arginine negative, whereas the so-called "colloid degeneration" droplets found in more or less necrotic areas (fig. 25) and hyaline casts, when present, are arginine positive. The tumor cells give positive reactions with the $AgNO_3$ test for vitamin C (fig. 26) and negative reactions with tests for lipase and for metachromasia.

When either progesterone, testosterone propionate, or DCA is given with stilbestrol, renal changes other than the cellular inclusions mentioned are often observed, *e.g.*, peritubular, spindle-shaped cells, and peculiar enlarged tubules (fig. 11) at the corticomedullary junction, and elsewhere in the cortex.

In the hamster kidney most fibroblasts, even those in the proximity of the proliferating tumor cells, show little or no alkaline phosphatase after prolonged treatment with stilbestrol. An occasional cortical fibroblast, however, may contain a relatively high concentration of the enzyme (fig. 31). After the removal of stilbestrol from an animal carrying a transplanted renal tumor, little or no decrease in the enzyme, as observed in histochemical preparations, is noticeable after 1 week; after 2 weeks, a slight decrease in the enzyme, indications of regression, and a decrease in the distinctness of cell outlines are apparent; after 3 weeks, some tumor cells still contain considerable enzyme, though most appear to have lost it entirely.

Mitochondria are fairly abundant; most of them are long, slender, slightly wavy filaments much more delicate in appearance than the long, thick, straight mitochondrial rods characteristic of the basal cytoplasm in the cells of proximal tubules. The Golgi apparatus is of medium size and lies between the nucleus and the apical pole of the cytoplasm; it appears as a fairly dense, compact network (fig. 27). Yellow, cytoplasmic inclusions, of undetermined nature, sometimes occur in regressing tumor cells.

A particularly peculiar feature of the renal tumor is the frequent presence of many ciliated cells. These cells have not been seen in all tumors, nor in all areas of single tumors; neither do they appear to constitute a majority of tumor cells in most microscopic fields. They are frequently seen as apparent crescents, forming low, simple cuboidal, epithelial layers, partially enclosing, within their concavities, plugs of ciliated and nonciliated cells (figs. 28 and 29). The crescents appear to constitute cross sections through collapsed cortical tubules, which may, upon careful search, be found sectioned longitudinally. The ciliated cells have a relatively acidophilic cytoplasm and deeply staining nuclei, usually with no nucleoli. Ciliary length may exceed cell height. Ciliary basal bodies are prominent (fig. 30) and are rich in alkaline phosphatase. The ciliated bands, unlike brush borders, exhibit metachronic waves. Since the majority of cilia face mere slits, or minute cavities, they are easily overlooked, even under oil immersion. It is quite possible, therefore, that they are present in far greater numbers than the routine examination of histological, and even of cytological, material under the light microscope would lead one to suspect. The apparently nonciliated cells are slightly larger, with basophilic cytoplasm and larger, more lightly staining nuclei.

Prolonged search of control and experimental material has revealed two instances of ciliated cells within recognizable renal tubules. These are in apparently intact distal convoluted tubules; one from a male treated with stilbestrol for 410 days (fig. 32), the other from a female treated for 397 days, starting on the 18th day of life. No renal tumors were found at autopsy, but upon sectioning the kidneys microscopic tumors were observed in each animal; in the male, one of the tumors was traced to the immediate proximity of the ciliated tubule, but no actual continuity with it was detected.

In addition to ciliated and nonciliated and cuboidal and spindle-shaped tumor cells, other instances of morphological variations are described and illustrated in figures 33, 34, 35, and 36.

DISCUSSION

The macroscopic observations described are essentially similar to those reported more briefly in other papers (*1*, *4*, *16*, *17*). Excellent black and white photographs of tumor-bearing kidneys and of metastases are available in these and other publications; Horning (*18*) includes a color plate illustrating tumor-bearing kidneys *in situ*, as well as a subcutaneously transplanted renal tumor. Of the microscopic observations, those pertaining to the general histological and histochemical characteristics of the tumor are similar to less detailed, earlier observations (*1*, *4*, *19*, *20*). It might be mentioned, however, that Horning and Whittick using the

Sakaguchi method for arginine report negative reactions; they also report negative results with the Millon method for proteins containing tyrosine, the ninhydrin-Schiff method for amino acids, the McManus-Cason method for preventing positive periodic acid-Schiff reactions by acetylation, and periodic acid-Schiff staining, due to failure of the blockade. None of these 4 methods were used in our studies. Horning and Whittick report negative results also with mucicarmine. They found that the tumor-cell lipides stain red with Nile-blue sulfate. Their observations on sections stained with Sudan dyes, with periodic acid-Schiff, and by methods for demonstrating glycogen and metachromatic substances agree with ours. Dontenwill and Eder (*19*), too, report the presence of alkaline and acid phosphatase in the tumor cells.

The occasional presence of amyloid in the kidneys of estrogen-treated hamsters has been reported frequently (*1*, *2*, *20*), but is incidental to tumor formation; most kidneys containing primary tumor nodules show no amyloid at all. In our colony amyloid has occurred more frequently in the non-tumor-bearing kidneys of estrogen-treated females than in the tumor-bearing kidneys of estrogen-treated males (*2*). Renal amyloidosis is not confined to estrogen-treated animals—it is present in hamsters with experimental leishmaniasis and tuberculosis (*21–23*).

The tissue edema characteristically associated with estrogen-induced stimulation of growth in the vagina and uterus (*24*) is absent, or relatively inconspicuous, in the proximity of even the earliest recognizable tumor nodules in the kidney of the estrogen-treated hamster. Also the abundant mast cells frequently found at the advancing margins of invasive tumors (*25*) are not characteristic of the growing margins of the dependent renal tumor, but in view of the reported decrease in mast cells after estrogen treatment (*24*) and the absence of mast cells in transplantable tumors of hamsters (*26*), this is not surprising.

Histogenesis of the Renal Tumor

As mentioned in the introduction, the estrogen-induced renal tumor of the hamster is usually referred to as a carcinoma and is considered to arise exclusively from some portion of the nephron, probably the proximal or the distal convolution. This interpretation is consistent with the general histological appearance of the neoplasm, and with certain tubular alterations observed in estrogen-treated hamsters; however, certain observations suggest that the histogenesis of the neoplasm may possibly be more involved than is generally supposed.

The only detailed description of the histogenesis is that of Horning and Whittick (*1*) who emphasize that, relatively early in the estrogen-treated male hamster, it is possible to observe numerous renal-cortical tubules exhibiting changes, consisting first of nuclear enlargement and loss of cytoplasmic eosinophilia. Progressive mitoses result in an eccentric clumping of nuclei and finally the tubular lumen may become obliterated by a compact mass of altered epithelial cells [fig. 6 and (*1*, *20*)].

It is reported (*1*) that further expansion of these intratubular masses is followed by rupture of the tubular basement membrane and extension of the proliferating epithelial cells between adjacent tubules.

A preliminary summary (*6*) postulates that growth pressures resulting from progressive hyperplasia, within the intertubular cell clusters, is followed by the collapse of portions of adjacent tubules, which are then converted into what appear in cross sections to be epithelial crescents partially enclosing cores of more hyperplastic cells. The concave surfaces of the simple epithelial layers constituting the "crescents" are ciliated, as illustrated first in 1950 (*4*). The summary includes the suggestion that the cilia arise *de novo* from the epithelial cells. Although tumors may arise sometimes from cells of the proximal convoluted tubules, it is not considered likely that the cilia of tumor cells represent modified brush borders. The two types of borders are quite different in various respects, *e.g.*, brush borders are periodic acid-Schiff and alkaline-phosphatase positive; ciliary borders are negative, except for the basal granules; the former are relatively thin and show nothing suggestive of metachronic waves. That the cilia do not correspond to the structure of the microvilli constituting the brush border, but are true cilia having an ultrastructure identical with that of cilia found in protozoa and in ciliated cells of different metazoan species, has been demonstrated by Manweiler and Bernhard (*27*) who consider the tumor to be exclusively epithelial in its origin. Since our material includes two examples of ciliated distal convoluted tubules in the hamster, it is highly probable that the ciliated tumor "crescents" are of tubular origin.

Cilia are not known to occur normally in any mammalian metanephric kidney, and we know of no other instance where they have appeared under abnormal conditions. However, the characteristic presence of cilia in the "neck" portion of the proximal tubule and in the intermediate segment of the metanephric nephron in certain lower vertebrates (*28, 29*) suggests that in the hamster (1) the "neck" portion of the nephron, between the simple squamous, parietal layer of the glomerular capsule and the definitive proximal convolution, may represent a survival of the ancestral reptilian neck segment; and (2) that, in spite of the presence of a thin segment in the hamster, there may be a survival of the reptilian intermediate segment in the distal portion of the tubule. The hormone sensitivity of part of the parietal epithelium of the renal corpuscle in the hamster (fig. 12) and the sex differences in this epithelium in the mouse suggest that, in these two rodents at least, a "neck" segment may be a genuine entity of the nephron, as it appears to be in lower vertebrates. The obvious experiment suggested by these considerations concerns the possibility of inducing tumors in the ciliated neck and/or intermediate segments of the nephron in lower vertebrates. The hormone sensitive (*30, 31*) sex segment (segment preterminal), first described in the reptilian kidney in 1903 (*32*) as corresponding to the mammalian distal convoluted tubule, is probably a collecting tubule and therefore of mesonephric origin (*30*); detailed histological and experimental studies of it (*33*) do not mention cilia. That a homologue of it survives in the hamster and is active in tumor histogenesis is unlikely.

The only suggestion in the literature that a mesonephric portion of the kidney might contribute to renal-tumor formation is our early one (*2*, *4*) that the localized band of sudanophilic epithelial cells in the lining of the renal pelvis might be implicated in some instances. Although sections are frequently observed which suggest such a possibility [fig. 6(*4*)], no clear-cut evidence supporting the suggestion is available. Neither have any tumors been observed in the ureters, urinary bladder, or urethra of any of our hamsters. It might well be recalled, however, as described and illustrated in another paper (*7*), that partially regressing tumor transplants may sometimes include areas of epithelium indistinguishable from that lining the ureter. Ising (*20*) also has observed a relationship between the tumor and the pelvic epithelium; she states, "Occasionally, the tumour growth originated in the medulla just under the mucosa of the renal pelvis." However, assuming the metanephric origin of the tumor cells, the presence of cilia in them, although unique in the mammalian metanephros, is not without counterparts elsewhere in the hamster, *e.g.*, the presence of ciliated cysts in the anterior pituitary, parathyroid, and thymus glands, and in testicular tubules (*34*).

In relation to the possibility of a connective-tissue contribution to the tumor, it is of interest that, in the area of the corticomedullary junction, where tumor nodules originate most frequently, connective-tissue cells (fibroblasts and macrophages) are far more abundant than elsewhere in the kidney (*35*). Also, the renal sarcomas, induced in hamsters by a mouse tumor agent carried in tissue culture, occur most frequently in this same area (*36*). These tumors, however, are quite different, histologically, from the estrogen-induced ones described here.

It has been reported that the administration of estrogen is followed by an increase in the content of alkaline phosphatase in certain fibroblasts. If some elements of the renal tumor were of fibroblastic origin, they might be expected to have a high concentration of this enzyme and tend to lose it after estrogen withdrawal. The preliminary histochemical observations described on the localization of the enzyme in renal-cortical fibroblasts and in regressing renal-tumor transplants, although not conclusive, are consistent with the concept of a possible stromal contribution to the tumor.

Still other observations suggest the possibility that the renal tumor may have a dual origin. Two distinct cell types, other than the ciliated and nonciliated ones described, may sometimes be seen arranged in intermingling cords or membranes, for example, in figure 33, from a kidney of a male treated with both stilbestrol and stilbestrol : cholesterol (1 : 4) pellets for 345 days, there may be seen a continuous mass of relatively small, deeply staining, tumor cells interrupted by irregularly distributed, apparently discontinuous masses of larger, lightly staining, more basophilic cells. Mitoses occur in each cell type. This arrangement is particularly interesting when considered in relation to the different manner in which sarcomas and carcinomas tend to invade normal tissues, as described by Barrett (*37*). According to Barrett, invading sarcomas form a continuous element or matrix in which the normal tissues are dispersed, while carcinomas appear dispersed in a continuous matrix of normal tissues. In terms of this concept the relatively small, deeply staining cells in figure 33 might be of stromal or vascular origin and the larger, more lightly staining cells, of tubular origin. Again, in figure 34, two distinct, and more or less segregated, cell types may be seen in a subpannicular transplant of the fourth serial passage in a previously hypophysectomized, stilbestrol-treated, male host. Here, too, mitotic figures occur in each cell type. In the papillary type of renal tumor [figs. 20 (*7*) and 36], recognized also by Horning and Whittick (*1*), two cell types are evident: one lining the irregular, cystic spaces and the other filling in the interstices between the spaces. Once more mitoses are present in each cell type. Cuboidal and spindle-shaped cells occurring within the same tumor nodule, too, frequently show a tendency toward segregation (fig. 35).

Wherever more than a single cell type has been recognized in the tumor, the morphologically different cells have been segregated into more or less distinct cell groups, *i.e.*, different cell types have not been observed to be heterogeneously intermingled. This tendency on the part of different cell types to adhere together, in more or less definite patterns, is interpreted as favoring the possibility of a multiple origin.

That at least one morphological type of renal-tumor cell is of epithelial origin is demonstrated by several observations readily made in estrogen-treated males, *e.g.*, (1) tubular alterations involving loss of cytoplasmic acidophilia, increased basophilia, increased cell height, increased mitotic rate, and nuclear clumping [figs. 5 (*4*), 6, 7, 8, 9, and 10]; (2) continuity of recognizable tubular elements with unmistakable clusters of tumor cells, with no intervening basement membrane (figs. 37 and 38); (3) occasional suggestions of tubule formation in tumor metastases from transplants [figs. 6 (*7*), 39, 40, and 41]; (4) occasional arrangement of tumor cells in membranes, resembling transitional epithelium [figs. 24 (*7*) and 25 (*7*)]; (5) the presence of ciliated cells (figs. 28, 29, 30, and 42); (6) prevalence of alkaline phosphatase in tumor cells and in proximal convoluted tubule cells, as contrasted with most intertubular connective-tissue cells (fig. 23); (7) cuboidal or columnar shape of the majority of tumor cells [figs. 13, 20, 21, 23, 28B, 34, 38, 39, and 9 (*7*)]; (8) the tendency of most tumor cells to be arranged in cords or plates enclosed by reticular fiber meshworks (fig. 14); (9) the loss of specific antigen by renal tubular epithelium during carcinogenesis (*38, 39*) and the corresponding loss, in the second cell generation, by renal tubular epithelium *in vitro* as determined by the uptake of I^{131} antibody by individual cells (*40*).

In view of these criteria, it is considered reasonably conclusive that the tumor includes a major epithelial contribution (carcinoma), in spite of the fact that the criteria are subject to some criticism: (1) The tubular alterations are not confined to estrogen-treated males. (2) Continuity between tubules and tumor could be due to secondary fusions. (3) Tubule formations within the tumor are not only rare, but never involve more than a small minority of the tumor cells. (4) There is no evidence that the tumor ever arises from transitional epithelium. (5) Although in higher organisms cilia are characteristic of epithelial cells, it has not been proved that cells of connective-tissue origin are incapable of acquiring them. (6) While it is frequently claimed that tumor cells tend to retain histochemically demonstrable enzyme systems, comparable with those of the cells giving origin to the tumors, exceptions are known to occur (*41*); also, while most stromal cells in the estrogen-treated kidney show little or no alkaline phosphatase, an occasional fibroblast contains a high concentration of it. (7) Cell shapes reflect environmental factors, *e.g.*, tension, as well as genetic constitution—under special conditions epithelial cells may become spindle-shaped and connective-tissue cells may become squamous, or cuboidal. (8) While it is true that the prevalent reticular fiber pattern of the tumor is that characteristic of carcinomas, it is true also that in areas consisting of spindle-shaped cells the pattern characteristic of fibrosarcomas and leiomyosarcomas (*42*) occurs; in such areas delicate reticular networks enclose each tumor cell instead of forming a common support for stratified groups of epithelial or epithelioid cells. (9) It may perhaps be questioned whether or not the radioautographic technique employed for the analysis of I^{131} antibody uptake by individual renal epithelial cells is precise enough to rule out the possibility of participation by the relatively sparse and inconspicuous stromal cells.

That the renal tumor may include a nonepithelial contribution is suggested by: (1) the frequent absence of continuity between tiny intertubular clusters of sudanophilic tumor cells and adjacent non-sudanophilic tubular cells; (2) the enclosure of single spindle-shaped tumor cells in reticular fiber sheaths; (3) the frequent occurrence of enlarged, peritubular, spindle-shaped tumor (?) cells in hamsters receiving either progesterone, testosterone propionate, or DCA in addition to stilbestrol; (4) the positive correlation between the location of early tumor foci and the relative concentration of macrophages and fibroblasts in the renal cortex; (5) the similarity between alkaline-phosphatase concentration in young tumor cells and occasional fibroblasts in stilbestrol-treated animals and the tendency for the enzyme to disappear after stilbestrol withdrawal; (6) the morphological relationships between the "ciliated crescents" (collapsed tubules?) and the partially enclosed "cores" of nonciliated (intertubular?) cells; (7) the occasional arrangement of tumor cells into continuous masses (sarcoma?) interrupted by more or less discontinuous masses (carcinoma?) of cytologically different tumor cells.

According to most orthodox morphological criteria the renal tumor is a carcinoma. In light of this discussion, however, and in view of the evidence presented in favor of a nontubular contribution, it is suggested that classifying the tumor on morphological grounds alone is difficult and may actually be inadequate. It should, therefore, be supplemented by data from other sources, *e.g.*, response of tumor cells to hormones known to affect certain epithelial and connective-tissue cells differentially; behavior in tissue culture; comparative histochemical studies.

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Plate 19

Figure 1.—Renal tumors, near corticomedullary border, in association with arcuate vessels. Ovariectomized hamster, implanted subpannicularly with stilbestrol pellet for 243 days. × 120

Figure 2.—Free-floating, intraperitoneal metastasis from primary renal tumor. Male hamster; subpannicularly implanted stilbestrol pellet 260 days. × 55

Figure 3.—Microscopic, intertubular nest of tumor cells stained with Sudan IV. Male hamster; subpannicularly implanted stilbestrol pellet 360 days. × 680

Figure 4.—Microscopic, intertubular nest of tumor cells stained with hematoxylin and eosin. Male hamster; 0.6 mg. of stilbestrol suspended in 0.2 cc. of 0.9 percent NaCl solution injected subpannicularly every other day for 410 days. × 1,100

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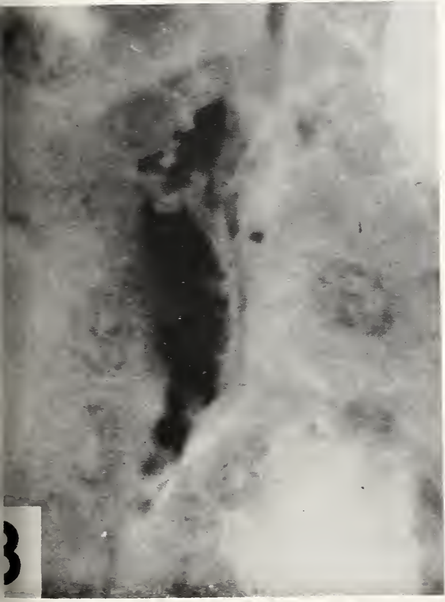

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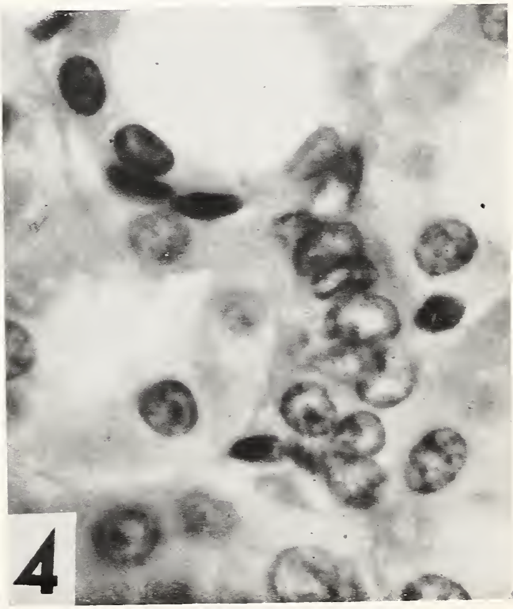

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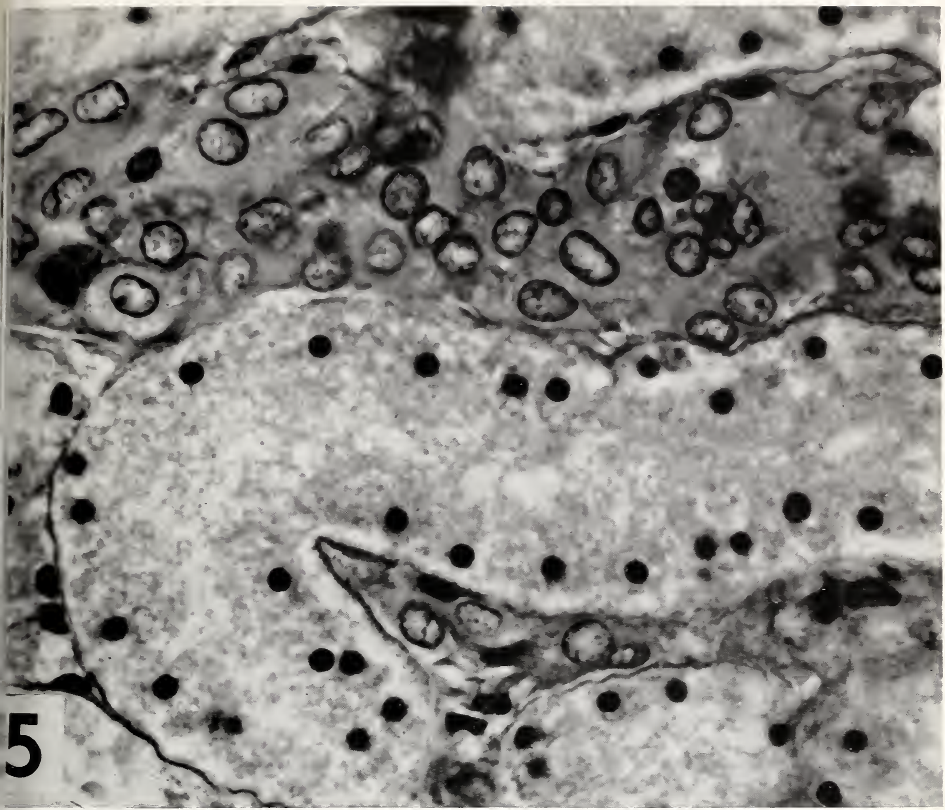
Plate 20

Figure 5.—Microscopic tumor, with intact cell nuclei adjacent to convoluted tubule, with pyknotic nuclei. Female hamster; testosterone propionate on the first day of life followed by a 20-mg. pellet of stilbestrol on the 50th day of life; died on 181st day of life; estimated 12 hours *post mortem* when fixed. × 720

Figure 6.—Nuclear clumping on one side of a distal convoluted tubule. Male hamster, implanted subpannicularly with stilbestrol pellet for 400 days; pellet removed 26 days before animal was killed. × 920

Figure 7.—Preludes to estrogen-induced neoplasia in the form of increased cell height and cytoplasmic basophilia. A. Distal convoluted tubule; the increase in number and thickness of stromal cells may be indicative of an early tumorigenic action of the estrogen on the cortical connective tissue. Male hamster treated as for figure 6. × 920



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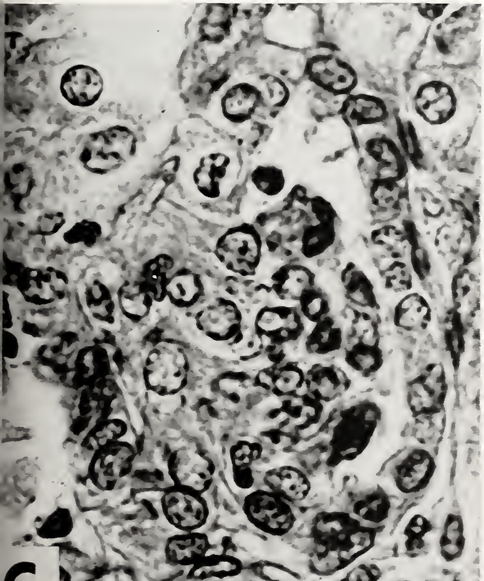

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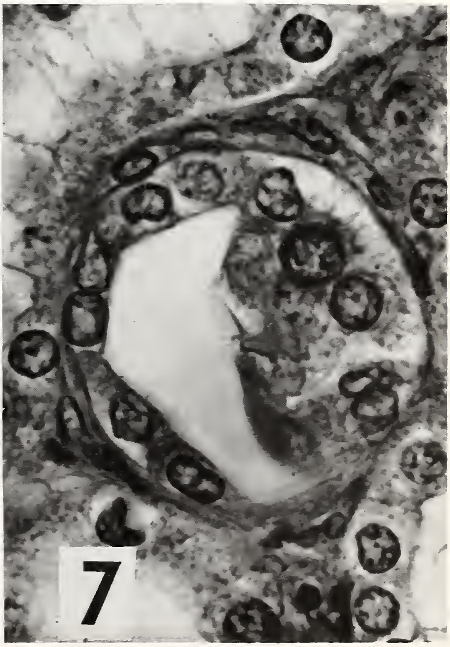

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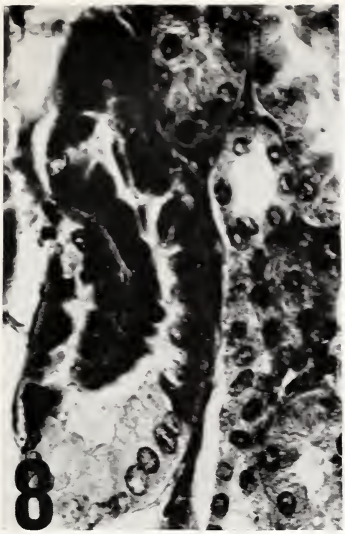
Plate 21

Figure 8.—Proximal convoluted tubule; the young tumor cells in the tubular loop differ from the adjacent normal proximal tubule cells also in that they are storing no trypan blue. Male hamster, implanted subpannicularly with stilbestrol pellet for 217 days, and receiving an injection of 0.5 cc. of 0.5 percent trypan blue in normal saline solution daily for 8 days before it was killed. [Reprinted by permission of *Cancer, see* (*4*).] × 465

Figure 9.—Nuclear clumping, with variations in nuclear size, in proximal convoluted tubule of an ovariectomized hamster; treated with stilbestrol pellet for 207 days and carrying the first passage of a metastasis from a primary renal tumor for 200 days. × 920

Figure 10.—Increased cell height, nuclear clumping, and nuclear enlargement in proximal convoluted tubule of female hamster with no renal tumors. Treated with stilbestrol and testosterone propionate for 223 days. × 1,380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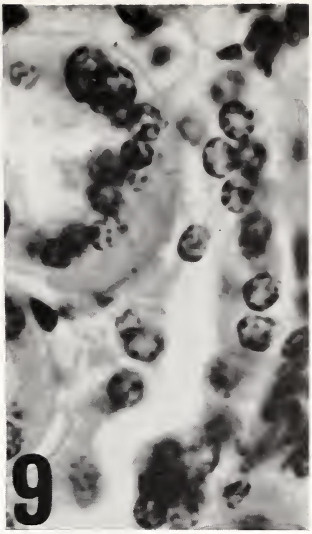
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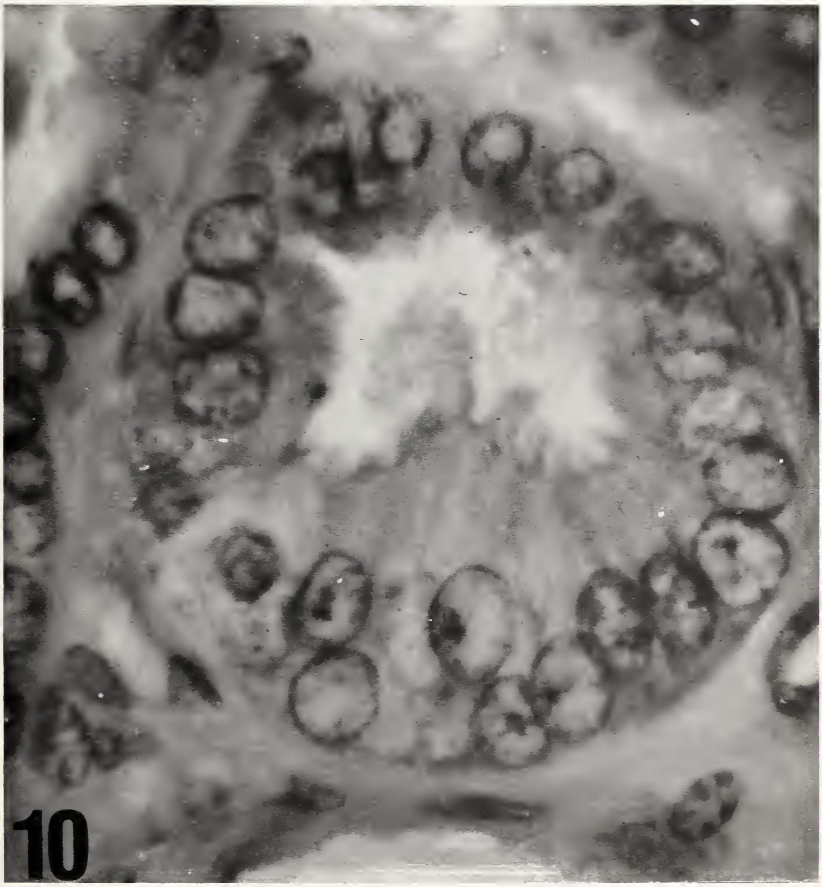
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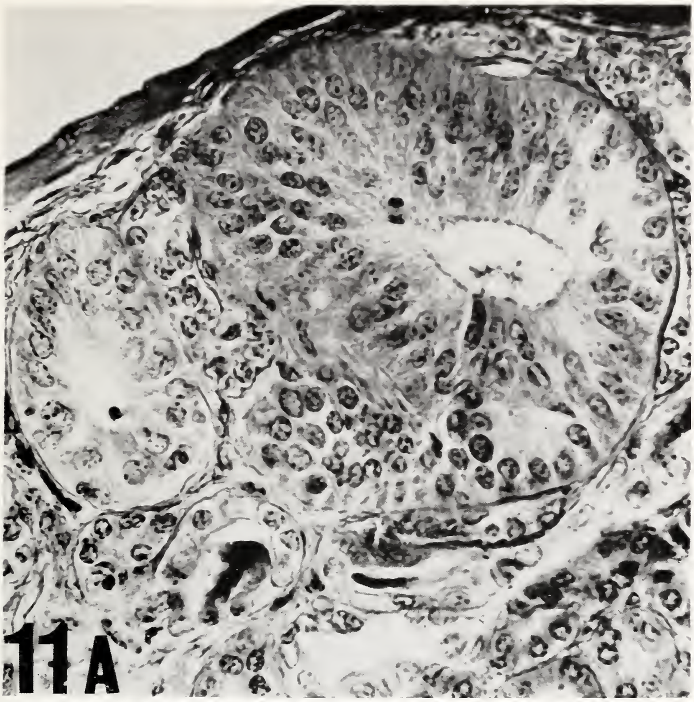
PLATE 22

FIGURE 11.—Abortive tumorigenesis in enlarged proximal convoluted tubules in male hamsters treated with a renal-tumor inhibitor and stilbestrol. A. Implanted subpannicularly with pellets of stilbestrol and DCA for 289 days. × 480. B. Implanted with stilbestrol and testosterone propionate for 434 days. × 920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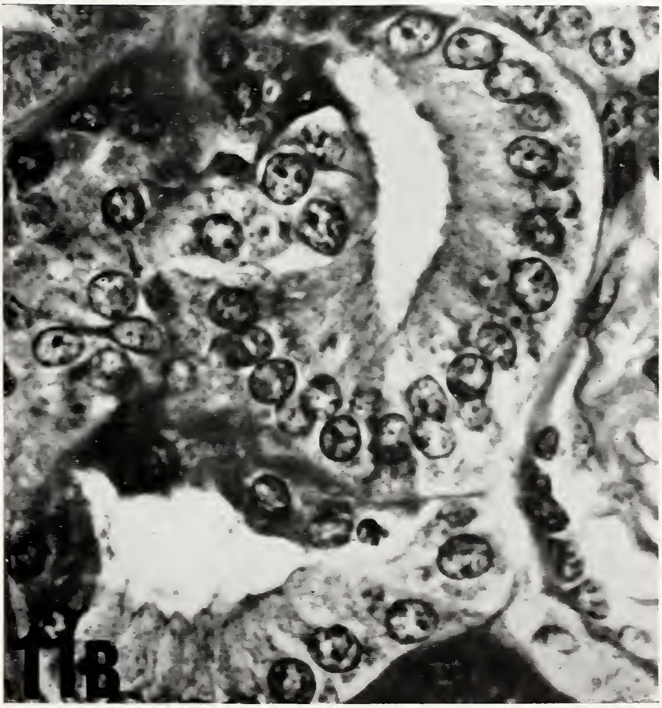
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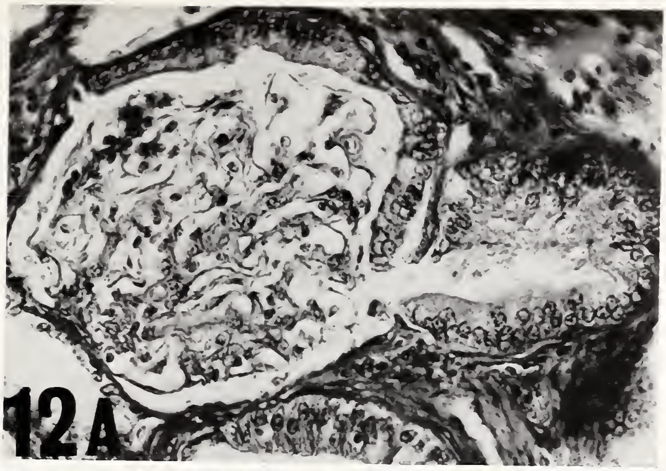
PLATE 23

FIGURE 12.—Increased cell height and basophilia in cells of parietal layer of glomerular capsule. A. Male hamster implanted with ethinyl estradiol for 301 days. × 340; B. Male hamster implanted with stilbestrol : cholesterol (1:4) for 300 days. × 500; C. Sudanophilia in thickened parietal layer of glomerular capsule. Male hamster treated with stilbestrol and testosterone propionate for 473 days. × 360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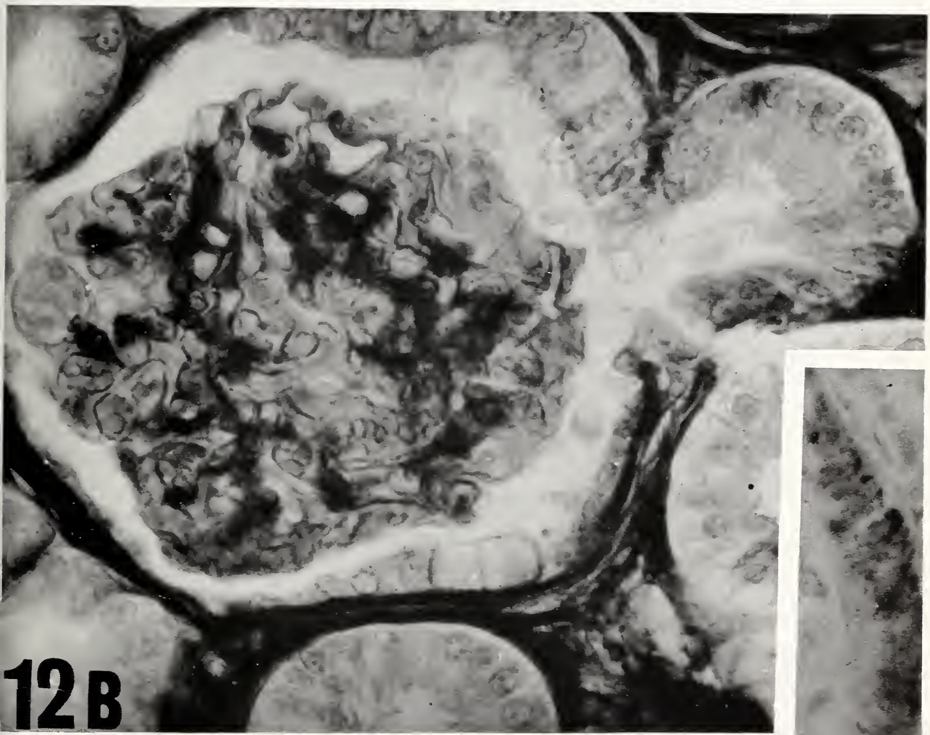
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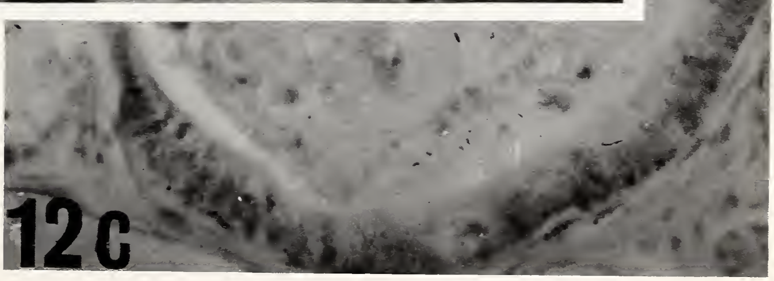
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PLATE 24

FIGURE 13.—Renal-tumor metastasis extending freely from the omentum into the abdominal cavity. The free surfaces are covered by a layer of mesothelium over a delicate connective-tissue stroma in which the macrophages are stained intravitally with trypan blue. Male hamster received an injection of 0.6 mg. of stilbestrol in 0.2 cc. 0.9 percent NaCl solution every other day for 360 days. × 1,000

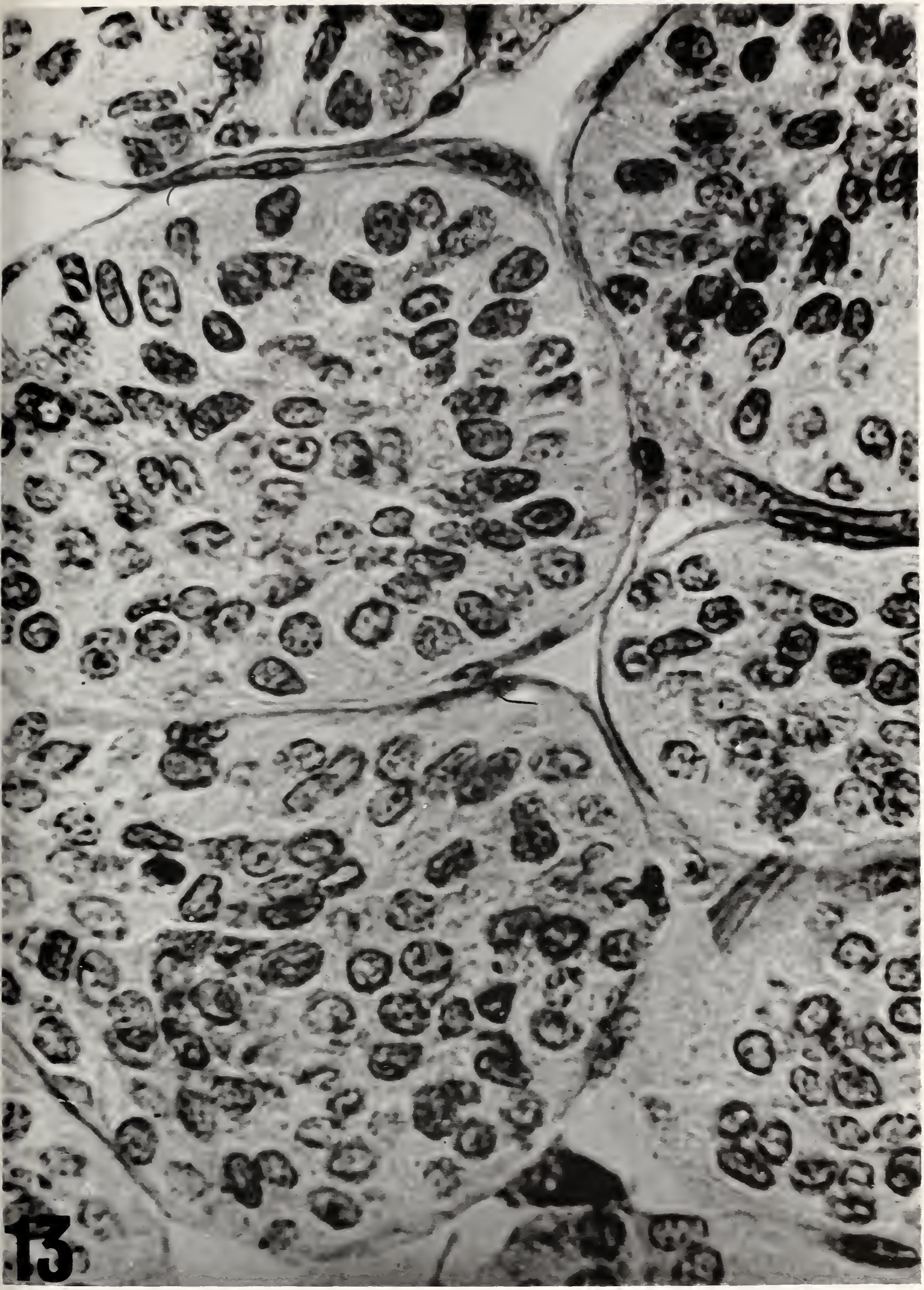

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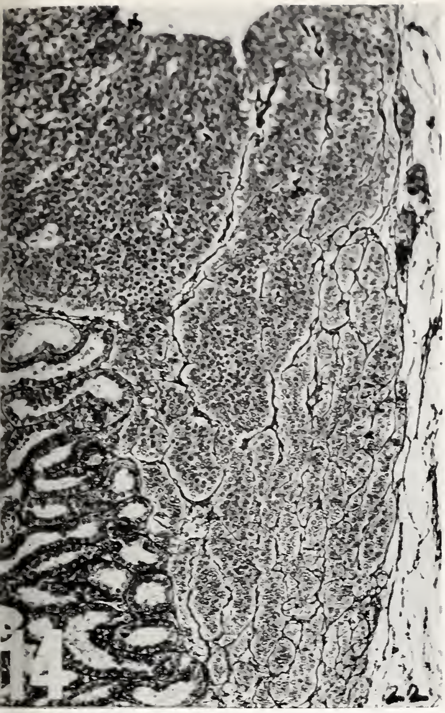
Plate 25

Figure 14.—Renal-tumor cells arranged in cords and plates of variable thickness, surrounded by delicate reticular fiber sheaths. Male hamster received an injection of 0.6 mg. of stilbestrol daily for 372 days. × 100

Figure 15.—Perithelial arrangement of renal-tumor cells around thin-walled vessels. Male hamster, treated as for figure 14. × 400

Figure 16.—Expansion of renal tumor into connective tissue of renal sinus. Treatment as for figure 14. × 100

Figure 17.—Spindle-shaped renal-tumor cells. Male hamster, treated as for figure 14. × 100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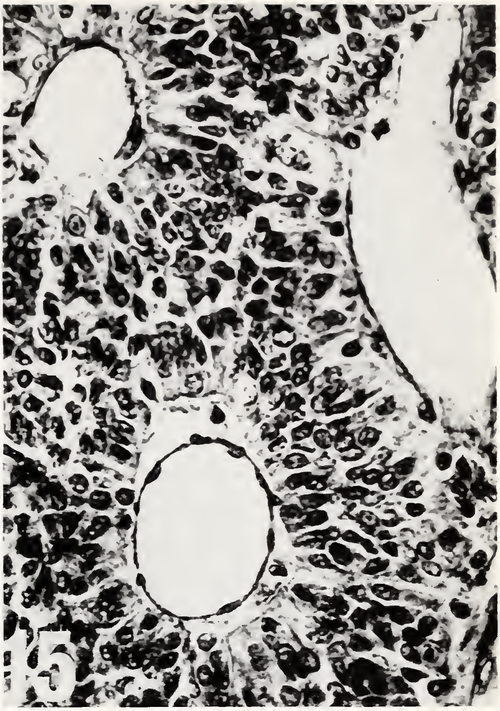
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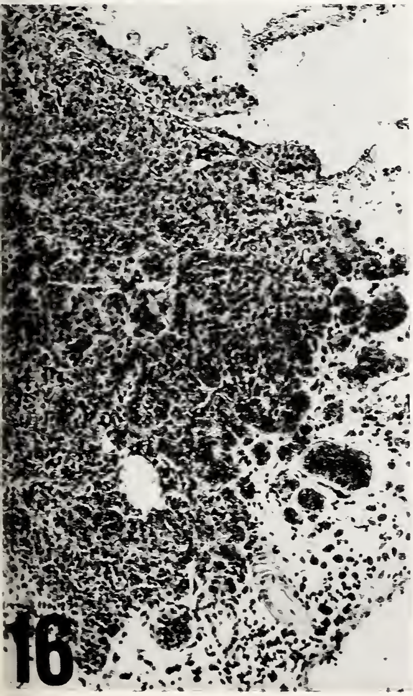
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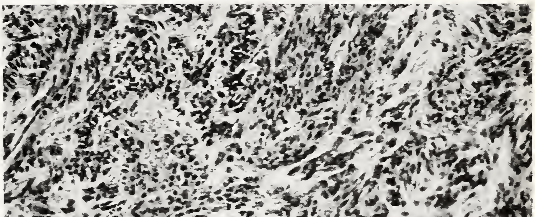
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PLATE 26

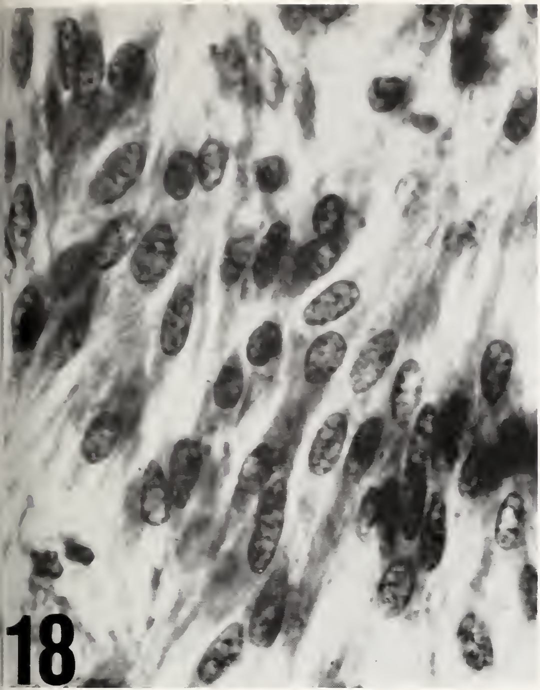
FIGURE 18.—Spindle-shaped renal-tumor cells in the seventh serial passage of a subpannicularly transplanted renal tumor. Male hamster, treated with tumor transplant and stilbestrol pellet for 117 days; during the final 16 days it was treated with a pellet of DCA also. × 920

FIGURE 19.—Reticular fiber sheaths about spindle-shaped renal-tumor cells. Male hamster, treated as for figure 14. × 900

FIGURE 20.—Chromosomal bridge in anaphase division of renal-tumor metastasis. Male hamster; injection of 0.6 mg. of stilbestrol in 0.2 cc. of 0.9 percent NaCl solution for 9 months. × 2,000

FIGURE 21.—"Signet-ring" cells in renal tumor. Treated as for figure 14. × 400

FIGURE 22.—Alkaline-phosphatase positive tumor in kidney of a male hamster implanted subpannicularly with pellet of stilbestrol for 280 days. × 46



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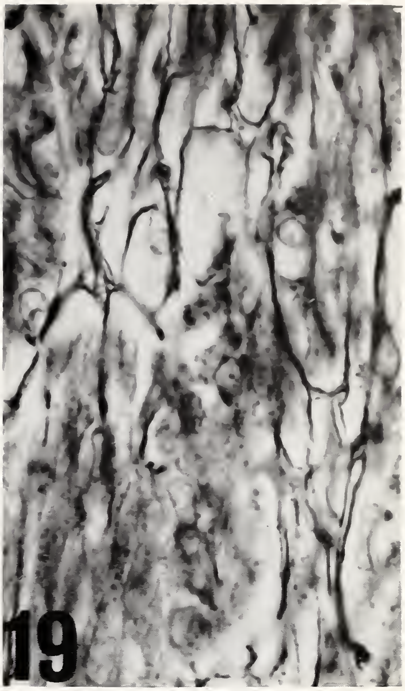

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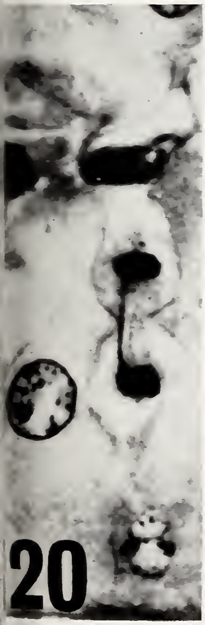

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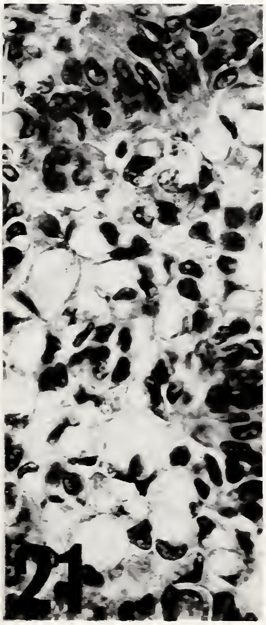

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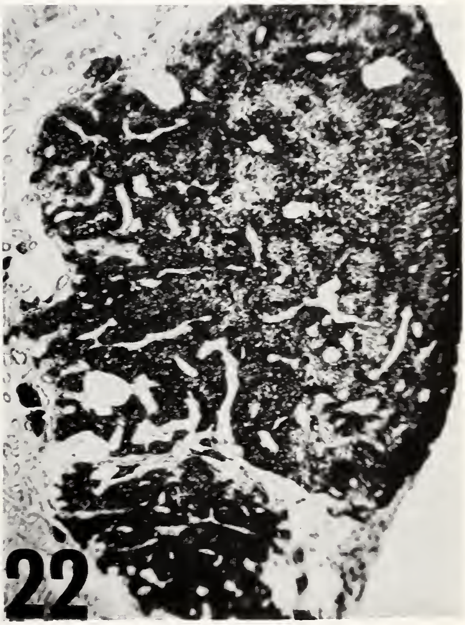

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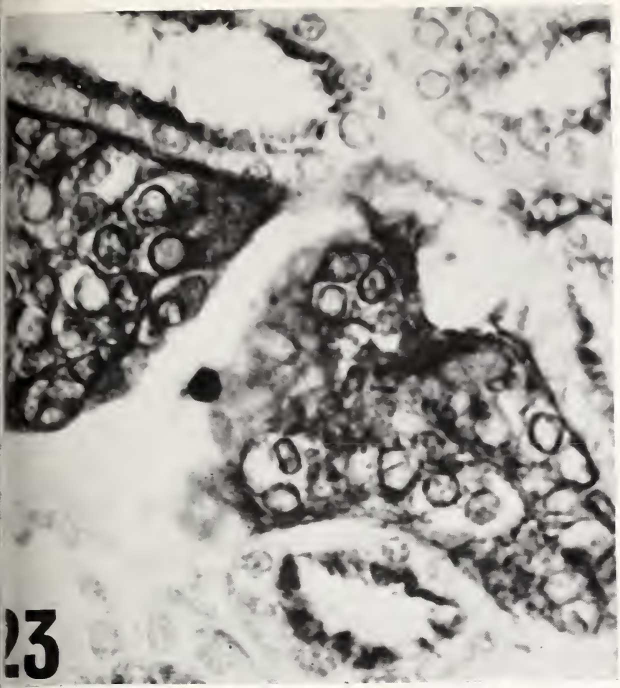
Plate 27

Figure 23.—Alkaline phosphatase in renal tumor and in brush border of proximal convoluted tubules. Treatment as for figure 22. × 720

Figure 24.—Acidophilic, globular, cytoplasmic inclusions in renal-tumor cells, similar to those of Russell body cells, in seventh serial passage in 227-day-old male host carrying transplant and stilbestrol pellet for last 196 days, and DCA pellet for final 120 days of life. × 1,080

Figure 25.—"Colloid degeneration" droplets in necrotic central area of renal tumor. Treatment as for figure 14. × 400

Figure 26.—Positive $AgNO_3$ test for ascorbic acid in kidney-tumor metastasis. Male hamster; stilbestrol pellet implanted for 405 days. × 920



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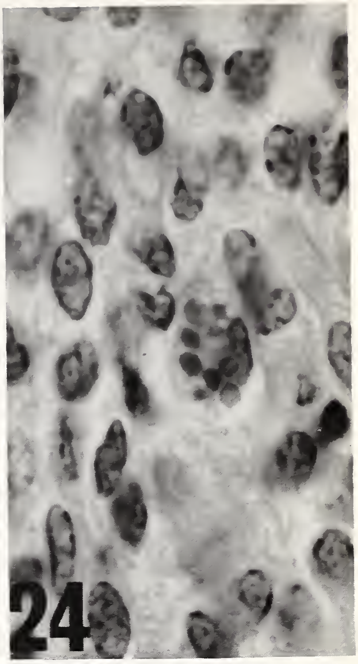

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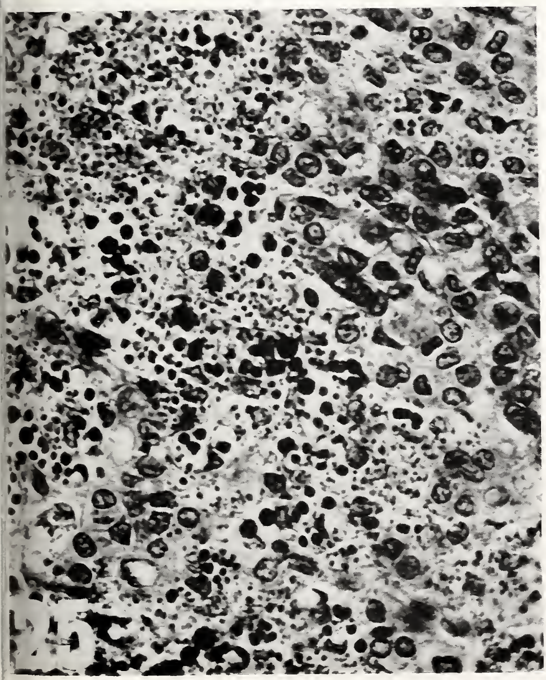

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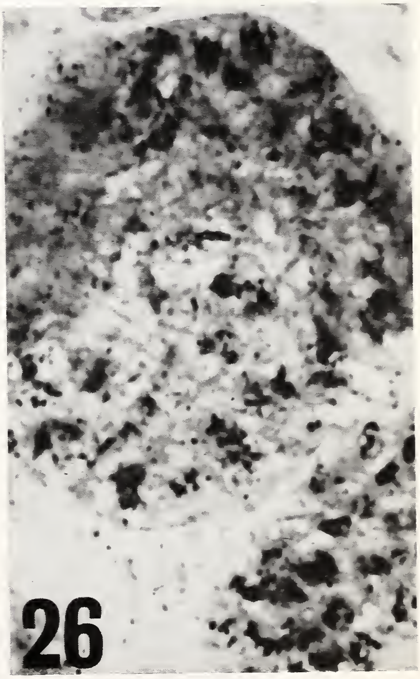

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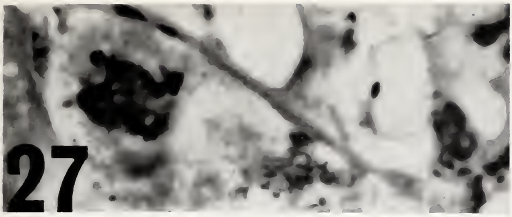
Plate 28

Figure 27.—Golgi apparatus in renal-tumor cells. Male hamster; stilbestrol pellet implanted for 380 days. × 2,000

Figure 28.—Ciliated crescents of simple cuboidal epithelium partially enclosing cores of apparently nonciliated cells, with more basophilic cytoplasm; scrotal metastasis from primary renal tumor; male hamster; 0.6 mg. stilbestrol in 0.2 cc. of 0.9 percent NaCl solution every other day for 331 days. A. × 400; B. × 850. [B, reprinted by permission of *Cancer, see* (*4*).]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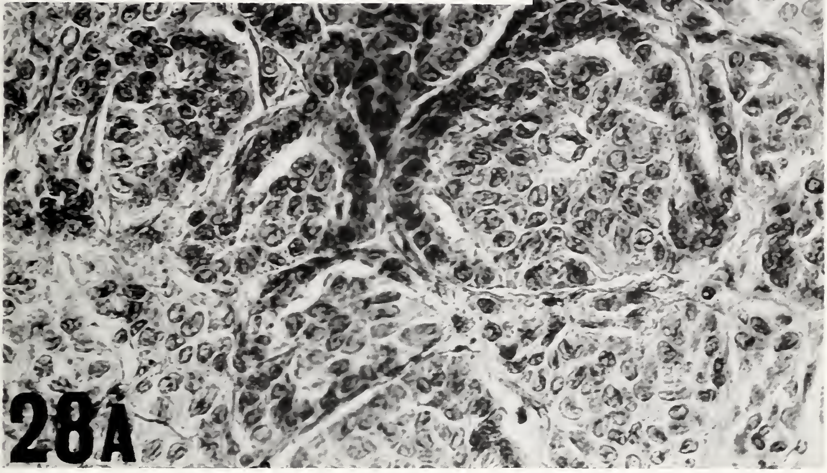
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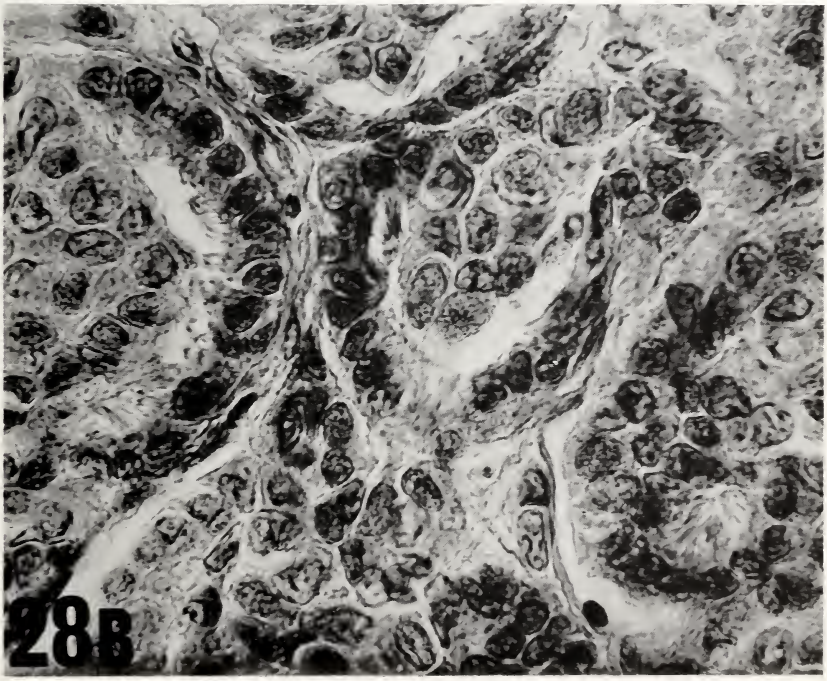
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Plate 29

Figure 29.—Same as for figure 28, except that the area illustrated is from a primary tumor in the left kidney. × 850

Figure 30.—Ciliated renal-tumor cells showing heavily stained basal granules. Hepatic metastasis from primary renal tumor. Male hamster; implanted with pellets of stilbestrol and of stilbestrol : cholesterol (1:4) for 345 days. × 2,800

Figure 31.—Alkaline phosphatase in fibroblasts near the corticomedullary junction of a male hamster. Treatment as for figure 22. × 900

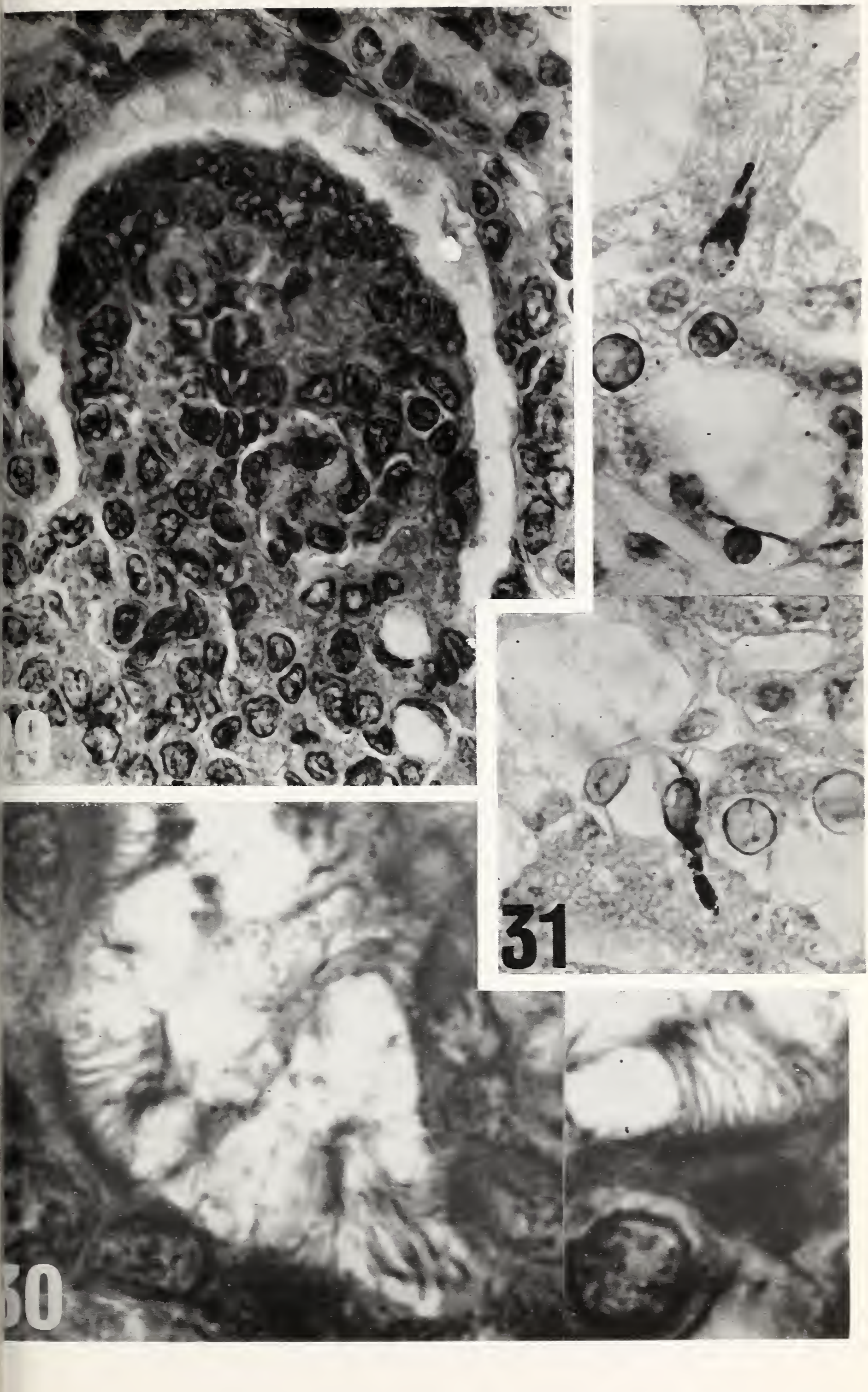
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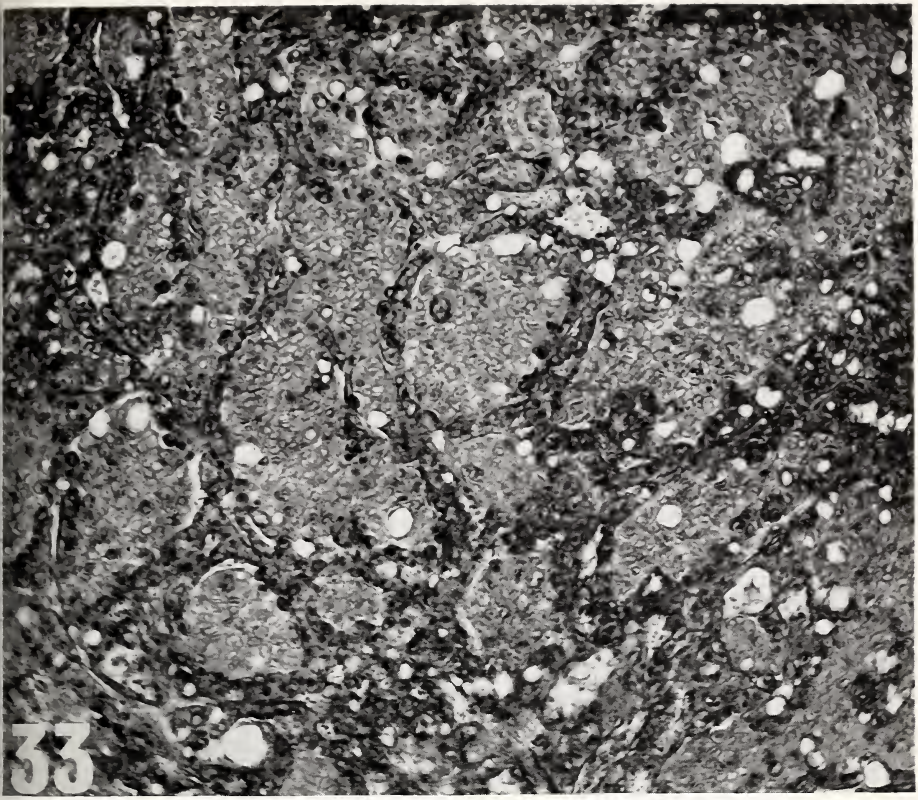
Plate 30

Figure 32.—Ciliated distal convoluted tubule, contiguous to, but not continuous with, a microscopic renal tumor. Treatment as for figure 3. × 2,900

Figure 33.—Two distinct cell types, arranged in intermingling cords, in a renal tumor. Male hamster; implanted with pellets of stilbestrol and of stilbestrol : cholesterol (1:4) for 345 days. × 160

Figure 34.—Two distinct, more or less segregated, cell types in the fourth serial passage of a renal tumor, in a hypophysectomized male host. Hypophysectomized and implanted with stilbestrol pellet for 166 days; transplanted for 105 days. × 240



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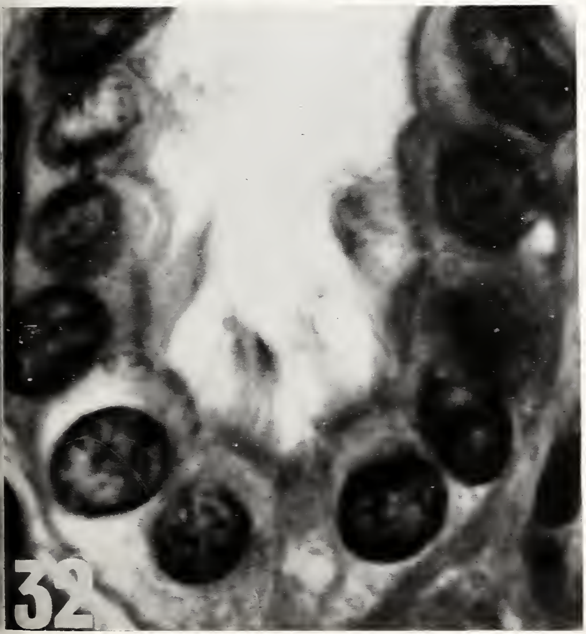

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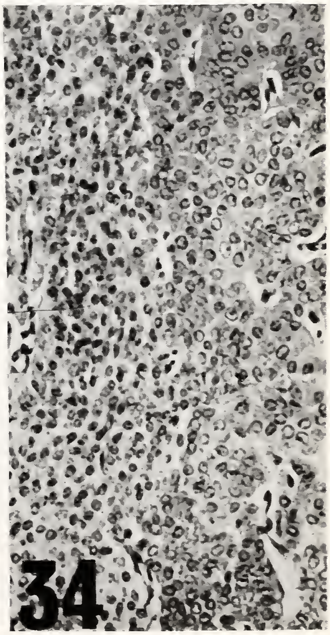

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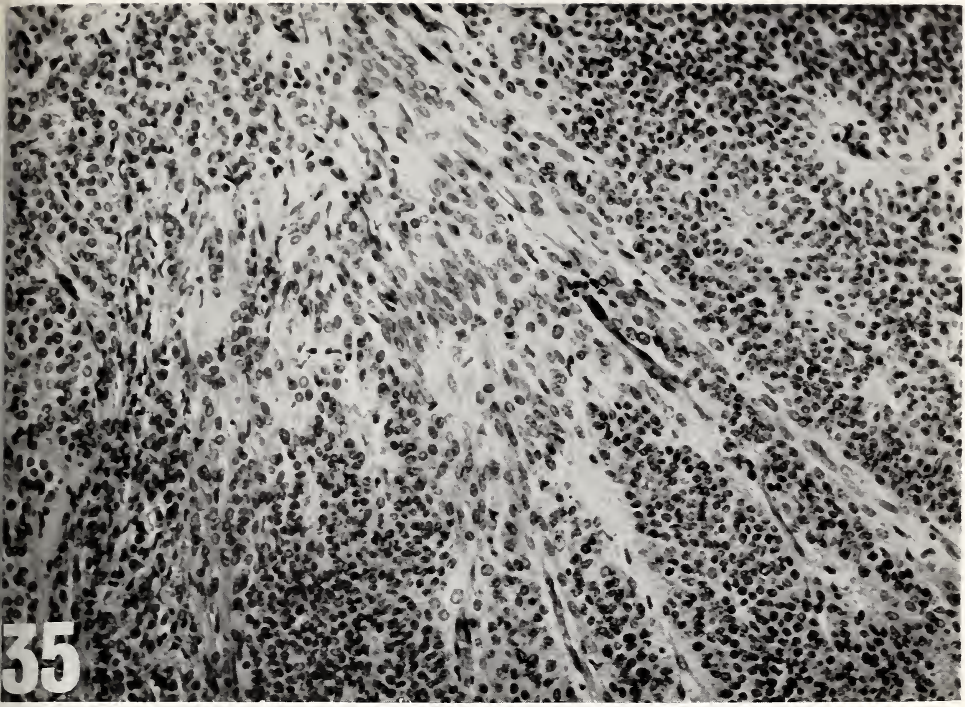
PLATE 31

FIGURE 35.—Two distinct, more or less segregated, cell types in the seventh serial passage of a renal tumor. Treatment as for figure 24. × 160

FIGURE 36.—Two distinct cell types in a multiloculated, papillary type of renal tumor. Simple columnar epithelial cells line the spaces, the interstices of which are filled with stratified layers of smaller, cuboidal cells. Male hamster; implanted with pellet of 7-methylbisdehydrodoisynolic acid for 404 days. × 120



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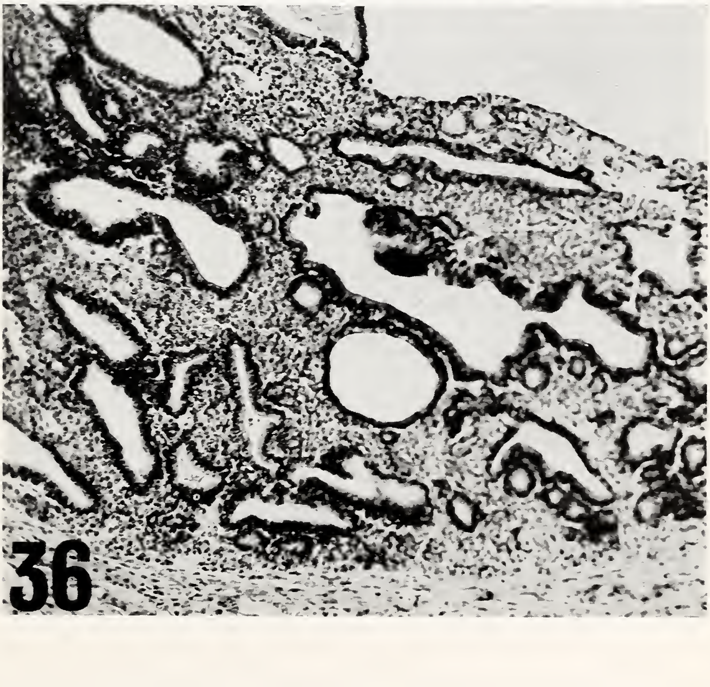

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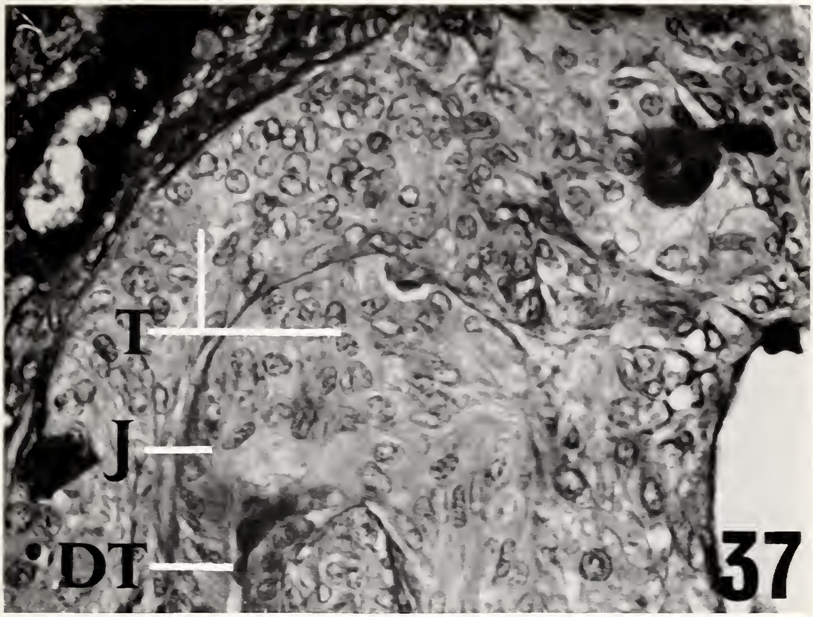
PLATE 32

FIGURE 37.—Continuity of renal tumor and epithelium of distal tubule. Male hamster; stilbestrol and testosterone propionate pellets implanted for 359 days. Incompletely hypophysectomized for 368 days; 0.3 mg. pituitary gland remnant found at autopsy; no involution of adrenal or thyroid glands. This animal is one of the rare ones in which the androgen did not completely inhibit the renal tumorigenic property of the estrogen. DT—lumen of distal convoluted tubule; J—junctional zone between epithelium of distal tubule and tumor; T—cords of tumor cells. × 400

FIGURE 38.—Continuity of renal tumor and epithelium of distal tubule through an interruption in the basement membrane. Male hamster. Estradiol pellet implanted in the spleen for 302 days. At autopsy a small adhesion was found between the spleen and the body wall. DT—lumen of distal convoluted tubule; J—junctional zone between epithelium of distal tubule and tumor; T—cords of tumor cells. × 480



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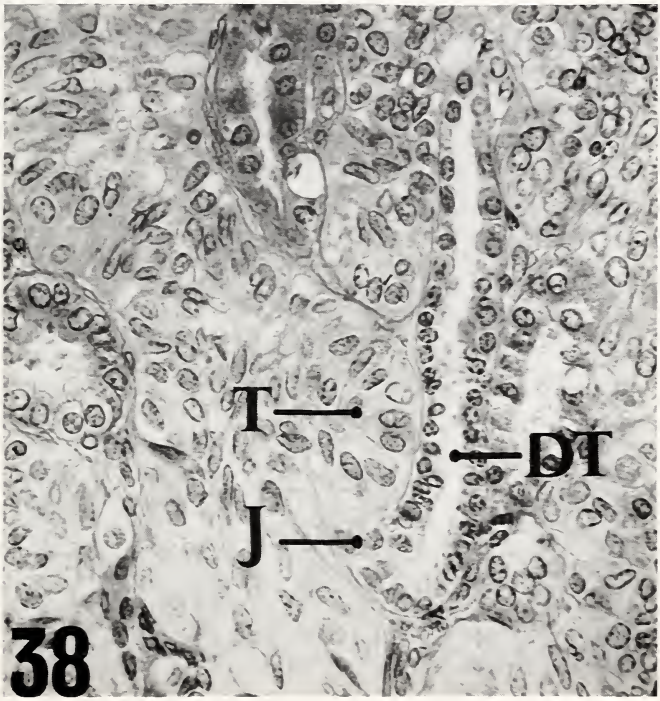

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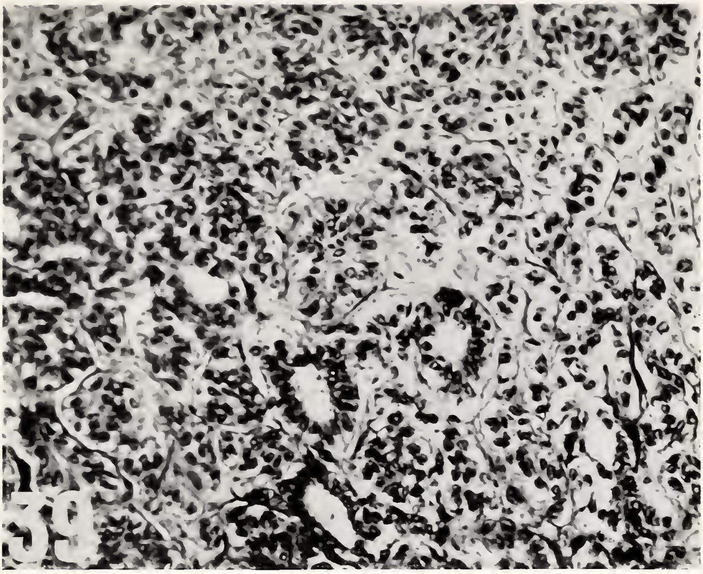
PLATE 33

FIGURE 39.—Tubule formation in a hepatic metastasis from a primary renal tumor. Male hamster; implanted with stilbestrol pellet for 437 days, replaced with testosterone propionate pellet for 184 days. × 240

FIGURE 40.—Tubule formation in a regressing epididymal metastasis from a primary renal tumor. Male hamster implanted with stilbestrol pellet for 484 days; animal killed 56 days later. × 240



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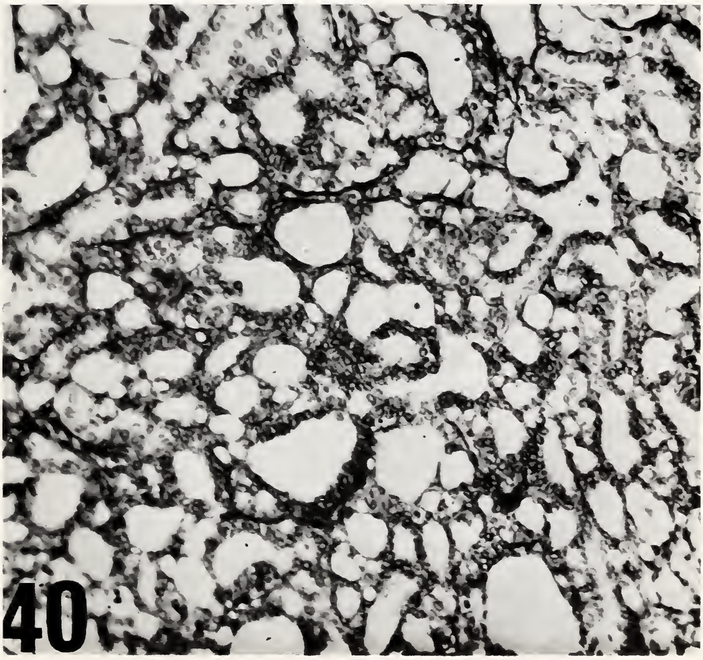

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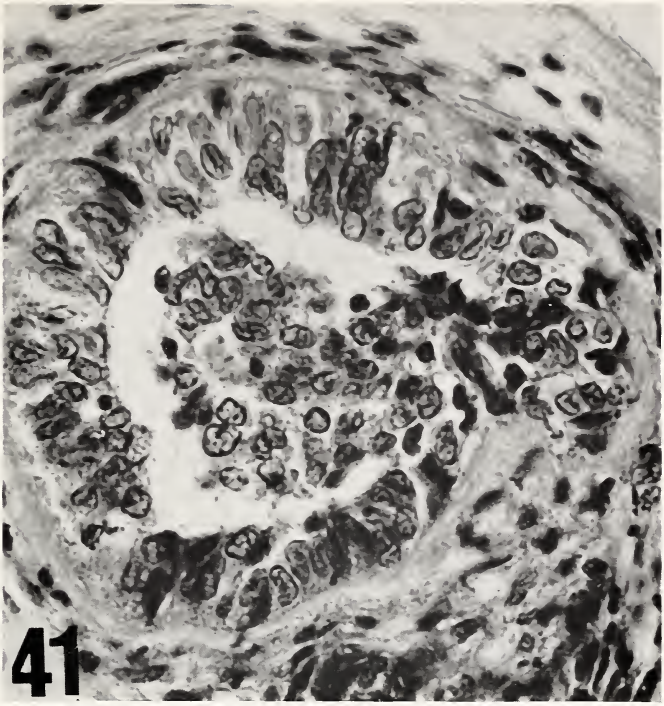
PLATE 34

FIGURE 41.—Tubule formation in the first serial passage of a renal-tumor metastasis in a hypophysectomized female host. Hypophysectomized and implanted with stilbestrol pellet for 90 days; transplanted for 75 days. × 730

FIGURE 42.—Ciliated simple columnar epithelium in an adrenal metastasis from a primary renal tumor. Male hamster; 0.6 mg. of stilbestrol in 2.0 cc. sesame-oil solution, injected every other day for 497 days. × 720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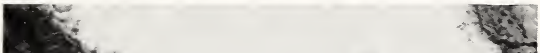
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U. S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
Public Health Service
National Institutes of Health

Symposium

ON NORMAL AND ABNORMAL DIFFERENTIATION AND DEVELOPMENT

ONAL CANCER INSTITUTE MONOGRAPH NO. 2

MARCH 1960

NATIONAL CANCER INSTITUTE MONOGRAPH NO. 2

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UNITED STATES OF AMERICA

Proceedings:
Symposium on Some Problems of Normal and Abnormal Differentiation and Development

Roscoe B. Jackson Memorial Laboratory
Bar Harbor, Maine
June 15 through 17, 1959

Honorary Chairman: Clarence C. Little, Director Emeritus
Roscoe B. Jackson Memorial Laboratory

Symposium Chairman: Nathan Kaliss, Assistant Director (Research)
Roscoe B. Jackson Memorial Laboratory

Edited by

Nathan Kaliss

Session I. Embryology and Genetics

Chairman: Leroy C. Stevens, *Roscoe B. Jackson Memorial Laboratory*

Speakers:

Clement L. Markert. *Biochemical Embryology and Genetics.*

Edgar Zwilling. *Some Aspects of Differentiation: Disaggregation and Reaggregation of Early Chick Embryos.*

Meredith N. Runner and Charles P. Dagg. *Metabolic Mechanisms of Teratogenic Agents During Morphogenesis.*

Biochemical Embryology and Genetics [1,2]

Clement L. Markert,[3] *Department of Biology, The Johns Hopkins University, Baltimore, Maryland*

The sciences of embryology and genetics are each concerned with a distinctive attribute of living organisms: embryology with the development of the individual, and genetics with the transmission of characteristics from one generation to the next. Nevertheless, in pursuing the ramifications of each of these disciplines, investigators eventually come to a common meeting ground where the basic questions are the same and where the initially distinct sciences of embryology and genetics merge into a single science of gene function. The role of genes in determining adult characters has always been a focus of interest in genetics, and many investigations have been made in the hope of elucidating the mechanisms by which genes govern the emergence of inherited characteristics. As a beginning, terminal characteristics have been described in biochemical or morphological terms and then their origins traced in stepwise fashion back to the earliest detectable expression of the responsible gene. Such efforts to unravel the consequences of genic activity have seldom proved successful and the nature of primary genic activity still eludes us completely. Nevertheless some progress has been made and, in the most rewarding investigations, adult characteristics have been shown to depend upon a specific type of molecule, generally a protein. This molecule or the details of its structure have in turn been correlated with the presence of a corresponding form of a single gene. Perhaps the best studied case is that of the human hemoglobins (*1*). A variety of hemoglobins, both adult and fetal, have been identified and the simple genetic control of their synthesis has been demonstrated. Each allelic gene for hemoglobin synthesis supervises the production of its own characteristic molecule differing from other hemoglobins in a single amino acid residue. In the hemoglobin of sickle-cell anemia, for example, the amino acid valine replaces the normally occurring glutamic acid residue at one point in the protein molecule, and this simple substitution produces serious physiological consequences. Clearly the structure of the gene

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[1] Presented at a symposium on normal and abnormal differentiation and development, held in celebration of the 30th anniversary of the Roscoe B. Jackson Memorial Laboratory, June 15–17, 1959, at Bar Harbor, Maine.

[2] These investigations have been supported by grants from the American Cancer Society, Inc., the U.S. Atomic Energy Commission, the National Science Foundation, and the National Institutes of Health, Public Health Service.

[3] The author wishes to thank Dr. Freddy Møller, Dr. Gabriel de la Haba, Frank Moyer, Harry Abramowitz, and Gershon Spector for their contributions, and Mrs. Ruth Creswell for her assistance.

is responsible for the corresponding structure of these protein molecules, but the mechanisms through which this responsibility is discharged are still unknown.

DIFFERENTIAL GENE FUNCTION

The clarification of the genetic control of hemoglobin structure is properly regarded as a major contribution to genetics, but hemoglobin synthesis is no less significant to embryology, for human beings synthesize two distinctly different types of hemoglobin: a fetal type characteristic of their embryonic life, and an adult type. Moreover, these two types of hemoglobin are controlled by independent genes. As development proceeds, the genes for fetal hemoglobin are suppressed and replaced in function by those for adult hemoglobin. Genetically controlled variants of fetal hemoglobin do not persist in the adult, nor are abnormal adult hemoglobins foreshadowed in fetal hemoglobin. All the genes affecting the fetal and adult hemoglobins appear to function completely independently. One set of genes operates in the embryo and an alternative set in the adult.

This example serves to emphasize the special and distinct interest embryologists must have in gene function, for they are concerned not only with gene function as such but with the additional and peculiarly embryological problem of why genes appear to function at one time and not another or function in certain cells while apparently remaining inactive in others. Although the mechanisms of cell division seem to insure that each daughter cell receives a full set of genes, these two daughter cells may develop very differently. One may become, for example, a pigment cell and the other a nerve cell. Such conspicuously different cells pose the old dilemma of how divergent development can occur in cells with identical genetic endowments. In facing this dilemma, both embryologists and geneticists are necessarily concerned with the central problem of present-day biology—the problem of gene function.

Divergent development in cells of identical genotype may be explained in either of two ways. First, despite identical genotypes, the functioning genome may vary, in which case cell properties would merely reflect the pattern of active genes. A second alternative explanation assumes that the functioning genome is the same in all cells, but the consequences of primary gene function vary in accord with nonchromosomal differences between the cells, differences arising out of initial heterogeneity built into the egg during its formation, and also arising out of the inevitable differences in environmental influences impinging on cells that occupy different positions in a cluster of cells. Either of these explanations or a combination of them provides an adequate basis for cell differentiation so far as genes are concerned.

Presenting the problem in this fashion seems realistic and meaningful today, but it is a very old formulation. Almost exactly 30 years ago when this laboratory was being established, Thomas Hunt Morgan (*2*), in his book *Experimental Embryology*, stated the problem in precisely this fashion. He commented then that no evidence was available to help decide between these alternatives. During the lifetime of this laboratory, has our position improved? I think it has, and I wish to present evidence in favor of the hypothesis that differential gene activation and/or inhibition lies at the foundation of cellular differentiation and therefore of embryonic development in general. In presenting this point of view I am mindful that plausible alternatives exist and that no critical choice among these alternatives is possible at the present time. Nevertheless, the marshalling of the evidence for one point of view may have at least heuristic value.

CELL-SPECIFIC GENE FUNCTION

If differential gene activation or inhibition lies at the foundation of cellular differentiation, then all genes cannot function all the time in all the cells of a metazoan. Considerable evidence has been obtained to support this conclusion. It is common knowledge that many enzymes, some of which are known to be under simple genetic control (*3*), are not uniformly distributed in all cells of an organism (*4*, *5*). Certain enzymes may be restricted to a single type of highly specialized cell. The enzyme tyrosinase responsible for the synthesis of melanin pigments in mammals and other organisms will serve as a well-known example. This enzyme appears to be restricted to the pigment-forming cells of the retina and to the migratory melanocytes. Other cells apparently have no use for this enzyme. When the genes for albinism are present, tyrosinase does not appear and no melanin is synthesized. However, this biochemical failure affects only the cells that normally make pigment. Other cells appear unaffected by albinism as one would expect, if the genes for tyrosinase synthesis normally never function in these cells.

Albinism in mice can be attributed most plausibly to a genetic incapacity to synthesize tyrosinase. However, in white spotted mice, the failure to form pigment must be explained in some other way, since the ability to synthesize tyrosinase is present in some cells of the body. An investigation has recently been initiated in my laboratory to analyze the pigmentation pattern in a recessive black-eyed white mouse. This mouse arose by mutation from the C3H strain and was generously made available to us by Dr. L. C. Strong. Tissues normally containing melanoblasts were removed from embryos of this mouse, and implanted intraocularly in albino hosts. Such implants from pigmented strains normally give rise to many melanocytes that migrate over the inner surface of the host eye (*6*). However, in no case did pigment cells appear in tissue implants from the white C3H strain. We can only conclude that the neural crest, the normal source of melanoblasts, did not contain cells capable of pigment formation, even in a favorable environment. Since the retina is pigmented in these white mice, the genetic capacity for tyrosinase synthesis must exist. We assume that the absence of pigment in the fur is due to a failure to activate the gene for tyrosinase synthesis during the differentiation of cells in the neural crest. This step in differentiation must therefore be responsive to a single gene that has no other obvious effects than to suppress the differentiation of melanoblasts. Of course a more general function may yet be found for this gene. In fact, pleiotropic effects of single gene changes are commonly observed, but even in these cases, only a few cell types are directly affected by their mutant genotype. Most affected cells would develop normally if transferred to a normal host.

TIME-SPECIFIC GENE FUNCTION

The evidence cited supports the thesis that the function of some genes is restricted to specific cell types; there is also evidence to show that genes function only during certain periods of development. Perhaps the best evidence comes from studies of the development of *Drosophila* embryos containing chromosome deletions. Poulson (*7*) has shown that Notch embryos, deficient for those regions of the X chromosome that are centered around the facet locus, fail to develop beyond the embryonic stage, though differentiating several normal tissues and organs despite the absence of many genes. Even more striking is the development of embryos bearing the scute deficiency. These embryos become fully differentiated larvae but die at hatching. Such examples could be multiplied, but they all show that extensive development can occur in the total absence of certain genes. We can only conclude that the missing genes have no important functions to perform up to the time of lethal crisis when their presence becomes essential for the next step in development. The periods of greatest sensitivity to genetic deficiencies commonly coincide with those developmental periods when new functions appear—*i.e.*, when the embryo becomes a feeding larva, pupates, or metamorphoses into the adult. This is exactly the result we would expect if new genes were being activated for the first time at these critical periods of development.

Biochemical investigations (*8*) and hybridization experiments (*9*) in amphibia also support the contention that many genes do not function during early stages of development. New biochemical synthetic activities do not appear until the cleavage stages have been completed, and the deleterious effects of a hybrid genome are seldom apparent before gastrulation. These observations suggest that the chromosomes (and genes) are replicating, and doing little else, during the early embryonic period of rapid cell division.

CHROMOSOMAL DIFFERENTIATION

If gene activation or inhibition occurs during development, there should be some corresponding physical or chemical change in the chromosome—*i.e.*, the chromosomes should differentiate. At least three types of evidence suggest that this is so: (1) Direct chemical analysis of nuclei and chromosomes from different types of differentiated cells reveals significant chemical differences (*10–12*). Of the four principal constituents of chromosomes, the deoxyribonucleic acid (DNA) and histone appear to be very similar in different cells. However, the ribonucleic acid (RNA) and residual protein vary considerably in amount between different cells and also within the same cell at different times. Perhaps the most striking changes in chromosomal composition occur during gametogenesis. These changes may be described in part as a simplification of the protein component of the chromosomes, and one is tempted to view these changes as a restoration of the undifferentiated state of the chromosomes in preparation for new development.

(2) The observations of Pavan (*13*) and Beermann (*14*) on chromosomal changes during the development of the larval tissues of certain insects demonstrate that chromosomal differentiation occurs in these insects. Their chromosomes display an intricate and characteristic pattern of enlarged bands and puffs that is specific for both the tissue and its stage of development. Probably the enlarged areas of the chromosomes indicate areas of more active function, but, in any event, the structure of the chromosome varies in accord with the stage of cellular differentiation.

(3) The work of King and Briggs (*15*) on nuclear transplantation demonstrates that the nucleus differentiates during development. These investigators have taken nuclei from frog embryonic cells at various stages of differentiation and transplanted them into enucleated frog eggs. The development of such eggs effectively measures the developmental capacity of the transplanted nuclei. The nuclei from cells in early stages of development proved equivalent to a fertilized egg nucleus; that is, normal animals developed from eggs equipped with such transplanted nuclei. When nuclei taken from cells at later stages of development were transferred to recipient eggs, such eggs developed into characteristically abnormal larvae. Moreover, the abnormalities were correlated with the state of differentiation of the cell furnishing the donor nucleus. Serial retransplantation of nuclei from such abnormal embryos elicited the same type of abnormal development in succeeding generations. These results suggest that the differentiated properties of the nucleus had become fixed and transmissible, despite

repeated nuclear division. Nonreplicating nuclear components would surely have been lost during repeated nuclear division, thus leaving only the chromosomes as the most likely repository of the differentiated characteristics of the nucleus.

The three lines of evidence just discussed, when taken together, provide impressive support for the hypothesis of chromosomal differentiation. Having discussed, so far, largely the work of other investigators, I should like now to turn to investigations from my own laboratory, which approach the problem of the genetic control of development on three different levels of cellular organization: chromosomes, enzymes, and cytoplasmic organelles.

DIFFERENTIATING NUCLEAR EXTRACTS

We assumed that cellular differentiation may involve the attachment of cell-specific proteins to the chromosomes. To examine this hypothesis, various protein fractions from adult frog and mouse cells were injected into fertilized frog eggs and the effects on development recorded. Several thousand eggs were injected. The following fractions were used: globulins, histones, and nucleoproteins from liver nuclei, and microsomes from liver cytoplasm. A volume of 0.066 μl. containing protein in amounts ranging from 0.5 to 0.005 μg. was injected into each fertilized egg before the onset of cleavage. Only those eggs that received an injection of the globulin fraction from frog-liver nuclei exhibited a consistent developmental arrest at the beginning of gastrulation. These arrested blastulae (about 200 have been produced so far) appeared normal except for the failure to gastrulate. The effective range of protein injected in the globulin fraction was 0.01 to 0.5 μg. Heating the globulin fraction (5 min. at 100° C.) destroyed its activity. Histological examination of sections, made from treated blastulae before development ceased, revealed normal mitotic figures and intact chromosomes. These observations suggest that the injected globulins produce subtle changes in the chromosomes of the developing frog embryo so that the chromosomes cannot fulfill their normal functions in development. We find these results exciting, but unfortunately we have not been able to obtain consistently globulin fractions that produce these characteristic effects. Some extractions yield effective fractions and others do not. Until we can eliminate this perplexing variability we cannot make much progress with this approach.

ENZYMES AND DEVELOPMENT

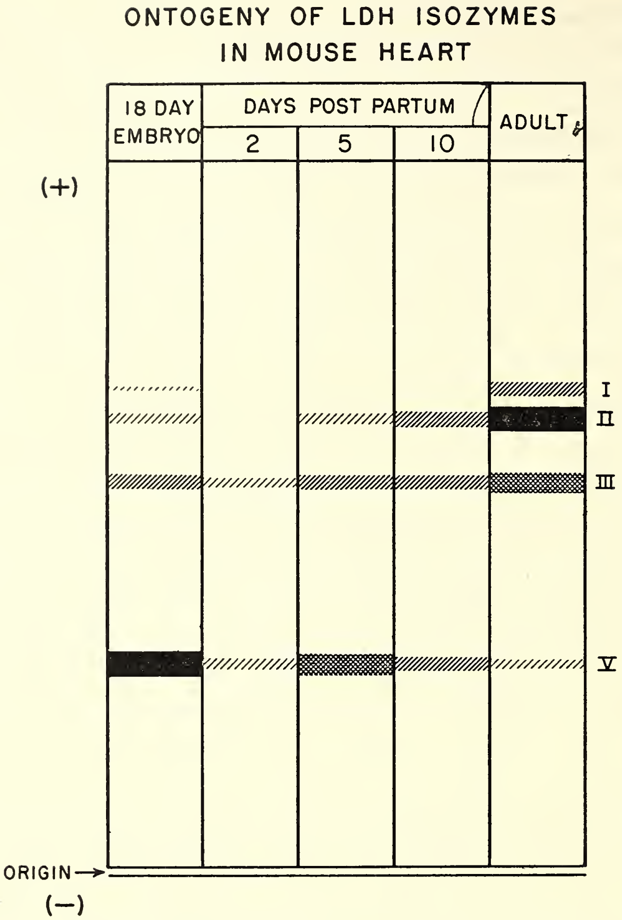
In biochemical studies of development, enzymes have frequently been the focus of attention both because of their importance in cellular metabolism and their close proximity to primary gene function. We also have investigated the activity of various enzymes during development and have observed a peculiar phenomenon for which we have no satisfactory explanation. Many enzymes have been shown to exist in several distinct molecular forms in the tissues of a single organism. We have called these separable molecules, having the same enzymatic activity, *isozymes*. In investigating the isozymes of lactate dehydrogenase (LDH) we find that the pattern of these LDH isozymes is species and tissue specific and changes during the embryological differentiation of the tissue (*16*). These isozymes are resolved from tissue homogenates by starch-gel electrophoresis. The pattern of LDH isozymes thus revealed in developing heart muscle of mice is shown in text-figure 1. It is apparent that the isozyme repertory changes during development. When other tissues are also considered, the generalization can be made that the pattern of isozymes may change during development by the disappearance of isozymes, the appearance of new isozymes, and most commonly by changes in the relative titer of the various isozymes.

This general pattern of LDH-isozyme behavior is also characteristic of the esterases, which are a large family of distinct but related enzymes with overlapping substrate specificities (*17*). The appearance of esterases during the postnatal development of mouse heart is shown in text-figure 2. An examination of this text-figure reveals that additional esterases appear as the tissue matures and the titer of individual esterases also increases, though the relative titer does not remain constant.

These changing patterns of esterases and particularly of the LDH isozymes pose important questions for both embryologists and geneticists. Does a single gene control the synthesis of the entire array of LDH isozymes or does each isozyme owe its existence to a separate gene? In view of the demonstrated genetic control of protein structure, it seems reasonable to support the second alternative. If this is so, then the pattern of isozymes may merely reflect the pattern of activated genes. However, it seems odd that an organism should require so many different molecular forms of the same enzyme, and the possibility should be investigated that the differences among isozymes may only reflect secondary modifications of protein structure attributable to the mode of attachment in the cell. In any event, isozymes appear to be a general and basic expression of the processes of cellular differentiation and as such deserve intensive investigation.

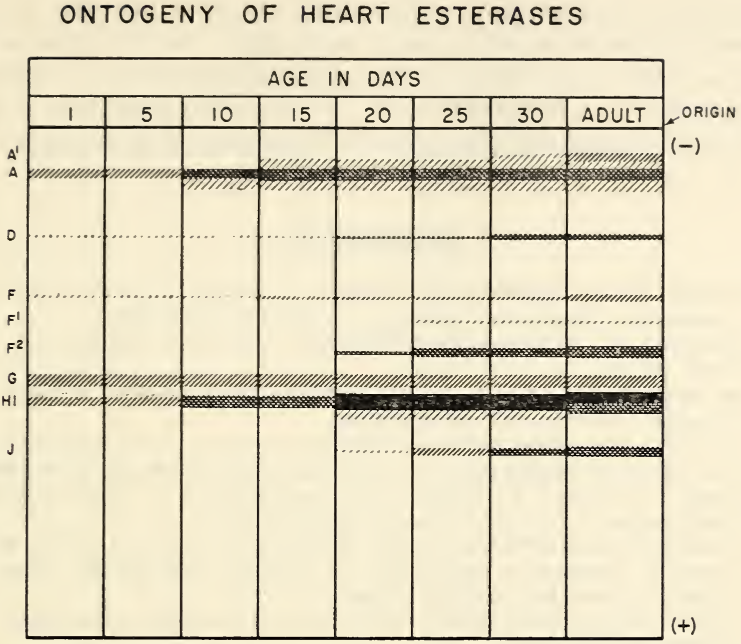
DEVELOPMENT OF A CELL ORGANELLE

All aspects of cell structure and function, as well as the relationships among cells, are subject to genetically controlled developmental processes, but very few cell organelles are accessible to combined geneti-



Text-figure 1.—The lactate dehydrogenase isozymes in heart tissue of mice at various stages in development are represented in this chart by bars. The size and density of the bars qualitatively represent the relative titers of the isozymes in tissue homogenates. The homogenates were resolved by electrophoresis in starch gel and enzymatic activity localized by a method previously described (*16*). Isozymes were found at four positions after electrophoresis, but the relative amounts and even the presence of certain isozymes change as the heart tissue matures.

cal-embryological investigation. One of the few that is easily studied is the melanin granule. The structure of the granule varies in different types of melanin-forming cells in the same animal and also varies in accord with the genotypic constitution of the melanocyte (*18*). My student, Mr. Moyer, is studying the structure of developing melanin granules by means of the electron microscope (*19*). He finds that the earliest evidence of the melanin granule is a diffusely granular material enclosed by a double membrane. This double membrane resembles in structure and dimension the membranes of the endoplasmic reticulum



TEXT-FIGURE 2.—This chart depicts zymograms of the postnatal changes in the esterases of mouse heart as observed after electrophoresis of tissue homogenates in starch gel. The general picture is one of a successive addition of new esterases and an increase in total esterase activity as the heart tissue matures. The methods used for visualizing these esterases have been described previously (*17*).

and also the double membrane enclosing the nucleus. Within this area osmophilic filaments appear and gradually increase in size until they coalesce to form the mature granule (figs. 1, 2, 3, and 4). Presumably the filaments are protein structures bearing the tyrosinase molecules. The increasing osmophilia would then parallel the increasing polymerization of the products of tyrosinase activity into melanin. It should prove instructive to examine whatever melanin granule precursors can be found in albino mice. The prevalent view would hold that only the tyrosinase molecules should be missing and these may not be essential to the appearance of the earliest form of the granule.

CONCLUSION

This brief survey of some problems confronting both embryology and genetics has placed the function of the gene at the focus of both fields. Development, however, is a never-ending process involving cyclic interactions at all levels of biological organization. These interactions have both structural and biochemical expressions, and both morphological and chemical investigations should prove fruitful at every step along the pathways of these interactions. The genes occupy a basic and indispensable position, and without their participation the dynamic interactions that drive development soon cease. As development proceeds, the interacting entities, including the chromosomes, are themselves changed (they are inseparable from the reactions) and these progressive changes are the essential content of embryonic differentiation.

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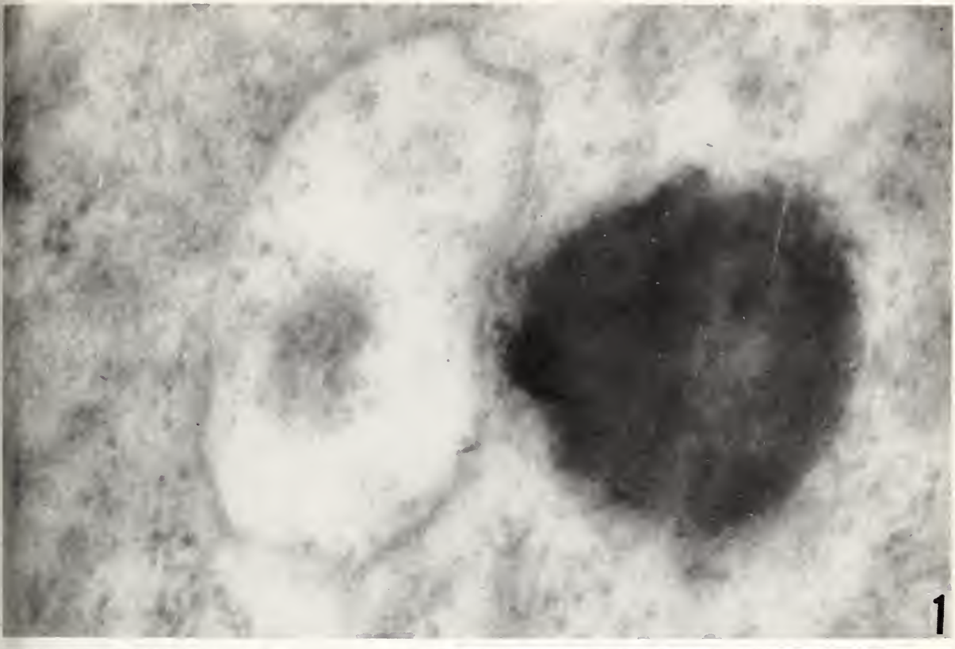
PLATES

PLATE 1

FIGURE 1.—Electron micrographs of developing melanin granules from pigmented retina of a 1-day-old mouse. The earliest appearance of the melanin granule is a diffusely granular material enclosed by a double membrane.

FIGURE 2.—*Note* filamentous structure of the developing melanin granule as seen in longitudinal section.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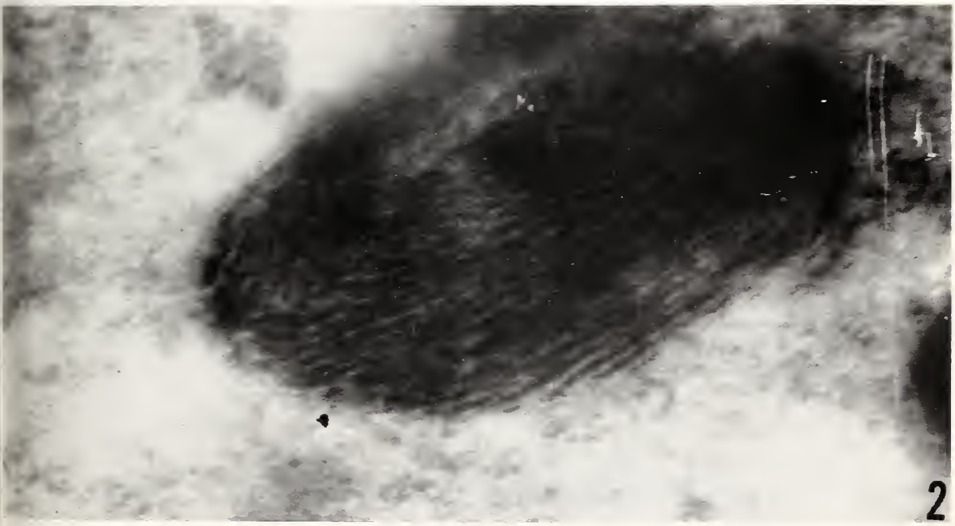
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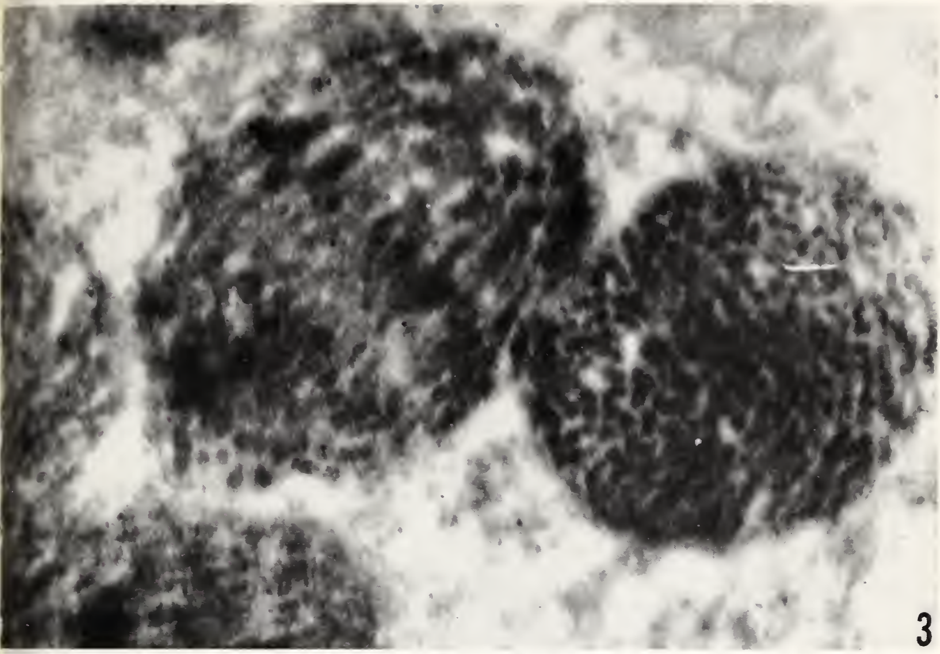
PLATE 2

FIGURE 3.—Electron micrographs of cross sections of developing melanin granules from pigmented retina of a mouse.

FIGURE 4.—In these cross-sectional views *note* that osmophilic filaments appear to expand and coalesce to form mature granule. Peripheral portion of granule still contains uncoalesced filaments.



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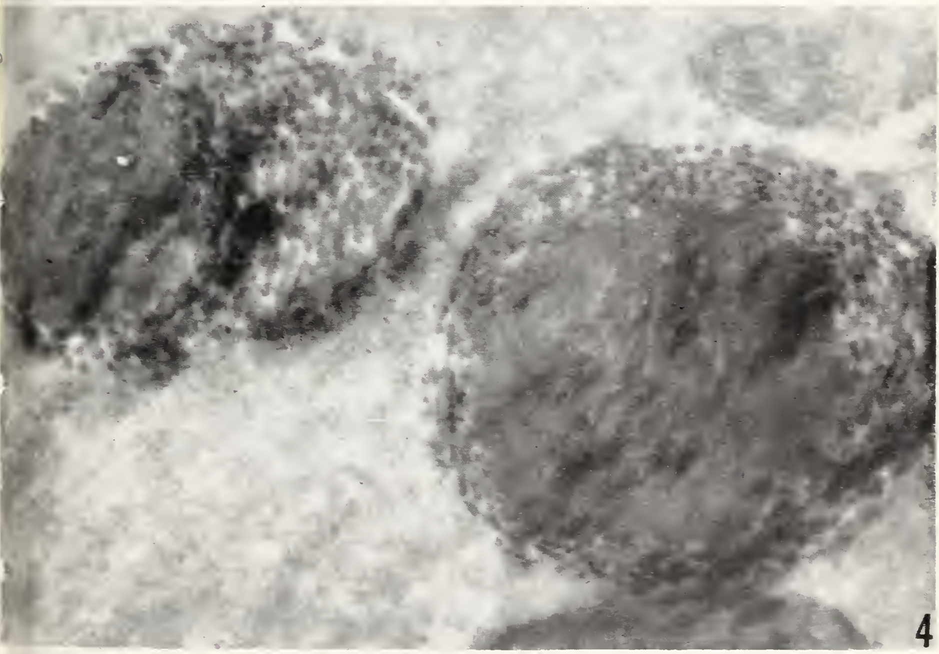

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ARKERT

Some Aspects of Differentiation: Disaggregation and Reaggregation of Early Chick Embryos [1, 2]

EDGAR ZWILLING, *Department of Biology, Brandeis University, Waltham, Massachusetts*

THE original topic suggested to me by the Symposium Chairman was "Ontogenesis of Differentiation." This appeared to involve the history of differentiation, in its many aspects, during the course of development of an individual organism. The enormity of such a task impelled me to request the less pretentious (and more permissive) title above. A full discussion of differentiation should include morphological, behavioral, chemical, physiological, and developmental aspects of the processes involved. While we can rarely discuss one of these aspects without reference to some of the others, it is even more rare for us to be in a position to consider all these aspects for a given example of differentiation. I propose, in this paper, to restrict my attention to certain behavioral properties of early chick-embryo tissues and to correlate the behavior with the subsequent appearance of certain morphological entities.

Behavioral studies of developing vertebrate tissues and cells, in their modern form, stem from the pioneering research of Holtfreter with amphibian material. The activities of bits of tissues and cells under a variety of conditions were observed carefully. These studies led Holtfreter to conclude that, during the course of development, cells acquire certain kinetic properties which impose upon them given behavioral patterns and that these kinetic properties and the consequent behavioral patterns are subject to modification during the development of a given tissue. That is, they do not remain constant during the history of the tissue. The basis for these properties is not completely understood but may in part be due to and in part reinforced by such structural peculiarities as the surface coat, etc. Nevertheless, cell and tissue behavior is subject to observation, and, at a biological level which involves such parameters as affinities, disaffinities, and axial polarizations, a good deal of the architecture of an embryo may be related to the described kinetics.

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[1] Presented at a symposium on normal and abnormal differentiation and development, held in celebration of the 30th anniversary of the Roscoe B. Jackson Memorial Laboratory, June 15–17, 1959, at Bar Harbor, Maine.

[2] This investigation was done at the Storrs Agricultural Experiment Station, Storrs, Conn., with assistance from research grant RG–3924, National Cancer Institute, National Institutes of Health, Public Health Service.

Much of the success of this research and its subsequent elaboration by Townes and Holtfreter (*1*), who applied the technique of dissociation and reaggregation of tissues to neurulae, depended on the peculiar properties of amphibian embryo cells. Such cells are large, vary in size, and may have various initial distinctive characteristics, such as large amounts of endogenous nutrient material, a prominent surface coat, and more or less heavy concentrations of intracellular pigment. In fact, when Townes and Holtfreter applied their procedures to amphibian embryo dorsal-lip explants, the absence of such differentials was a handicap to a close tracing of the processes of dissociation and sorting-out during reaggregation. The possibility of regulative and inductive interactions in this still-labile tissue from the younger embryos was another handicap in an evaluation of the behavior of the cells.

The application of the techniques of tissue dissociation to amniote embryos was initiated in 1952, when two independent laboratories almost simultaneously published the results of experiments with chick embryos. Moscona and Moscona (*2*) and Moscona (*3*), working in Fell's laboratory, elaborated procedures whereby tissues from 3- to 5-day chick embryos could be converted to a cell suspension by the action of the enzyme trypsin. Such cells may subsequently reaggregate and their development may be followed in tissue culture. Weiss and Andres (*4*), then in Chicago, used mechanical means to obtain a cell suspension from tissues of 1-, 2-, and 3-day embryos. The suspended cells were injected into the blood vessels of older host embryos and were later, in a small percentage of cases, recovered as extra-embryonic teratomatous masses or as patches of pigment in the embryo or its accessory membranes.

Most of the details of reaggregation and subsequent sorting-out of cells cannot be followed in avian embryonic material because of the absence of distinctive differentials, which were noted for amphibian material. The difficulties encountered by Townes and Holtfreter with the dorsal-lip region of gastrulae are even more exaggerated in bird embryos. Nevertheless, many (possibly too many) of the properties of these cells have been inferred from the subsequent development of the reconstituted tissues. Recently Moscona (*5*, *6*) and Wilde (*7*) have combined cells from chick and mouse embryos (whose staining characteristics are sufficiently distinctive so that they may be recognized histologically) and have been able to clarify several important problems. Other techniques for following cells of amniote embryos are becoming available.

While the fascinating problems of the mechanisms involved in cell-to-cell attraction, sorting-out, and binding are beyond the scope of this paper, it may be well to outline briefly the impressions gained by the authors previously mentioned, as well as those from the recent study of Stefanelli and Zacchei (*8*). All these authors agree with these essential points: Initial reaggregation, after dissociation, is a random process which takes place irrespective of cell specificities. It is aided by random movements of cells and aggregates and by such factors as the nature of the medium, the shape of the vessel in which the cells reaggregate, the nature of its surface, and the presence of artifacts on that surface (scratches, fibers, etc.). After the formation of an aggregate, there is a sorting-out process which results in the reassociation and subsequent binding of like cells. The final organization of the reconstituted tissues in a heterogeneous mixture probably depends on the interaction of cells with different behavior properties. The mechanisms and forces responsible for the sorting-out and final cell-to-cell affiliations are little understood. [*See* recent review by Steinberg (*9*).]

Another avenue of investigation is concerned with the type stability of cells subjected to these various dissociative procedures. This subject is still controversial (*5*, *10*, *11*), but the bulk of current evidence, based largely on experiments designed to protect and stabilize the cells, indicates that there is a marked retention of the original specific properties of the cells. In the words of Weiss and Andres (*4*), "The emphasis lies on the contention that a cell of a particular type can only become lodged in specific 'niches' of closely circumscribed character to which it is physically, chemically and physiologically adapted." Whether or not this is the final conclusion may be decided by future experiments. However, it must be remembered that such a statement applies to relatively well-differentiated cells which have already acquired most of their specific properties. The evidence from a vast amount of research, concerned with tissue interactions of young embryos, virtually forces us to reverse this statement for immature tissues and insist that it is the "niches" where particular groups of cells find themselves which impose upon them the physical, chemical, and physiological properties characteristic of a particular type of cell.

It is with the behavioral properties of the cells and tissues of chick embryos of an intermediate stage that this study concerns itself. The definitive primitive streak (DPS) embryo has a nodal region which, according to many criteria, is reasonably stabilized in the direction of forming neural and various other complex tissues (*12*). Isolated regions posterior to the node level have little or no capacity to form axial structures. This fact was described by Willier and Rawles in 1931, verified by many other investigators, and recently received striking confirmation and elaboration by Spratt (*13–15*). These tissues are still labile and subject to organizational and inductive interactions. The peripheral portions of the pellucid area of such embryos contribute to the formation of extra-embryonic structures. However, they still have the capacity to respond to inductive stimuli (*16*). It is this system that has been studied by means of dissociation and reaggregation procedures.

MATERIAL AND METHODS

I shall not go into details of the techniques used. Embryos, or parts thereof, were dissociated in solutions of ethylenediaminetetraacetic acid (EDTA) and made alkaline (*ca.* *p*H 8.5) with KOH. Modifications of the techniques described in 1954 were used (*17*). (As a matter of fact, the techniques employed were subject to evolution and change during these experiments and shall be described elsewhere.) The EDTA was removed and the tissues were washed several times in a mixture of horse serum and Tyrode's solution and then dissociated more completely by means of fine pipettes. The cell suspensions were allowed to reaggregate spontaneously in the horse serum-Tyrode's medium in depression slides or depression dishes—so-called Grobstein dishes (*18*). Cell concentration was usually adjusted so that most cells reaggregated in 1 to 3 large masses. The reaggregation process was usually completed in 8 to 10 hours at 37.5° C.

The large reaggregates were cut into smaller pieces, usually *ca.* 0.25–0.3 mm. in diameter, which were then either grafted to a host site or continued in a tissue-culture situation. Chorio-allantoic grafts were made, as were grafts to the body wall of 2-day host embryos. The majority of the reaggregates were continued in tissue culture. They were cultured on plasma clots or, in most instances, on platforms whose lower surface was bathed by a fluid medium, usually embryo extract, horse serum, and Tyrode's solution in proportions of 2:1:1 or 4:3:3. Such platforms consisted of washed lens paper, either alone or coated with agar, or Millipore filters (150μ thick and 1.2μ pores). In all instances the platform was supported on a glass ring. Cultures were kept in gassed (5% CO_2 in air), moist chambers at 37.5° C. Medium was changed every 2 to 3 days and experiments were usually terminated when a total age of 10 to 12 days had been achieved. Routine histological procedures were used.

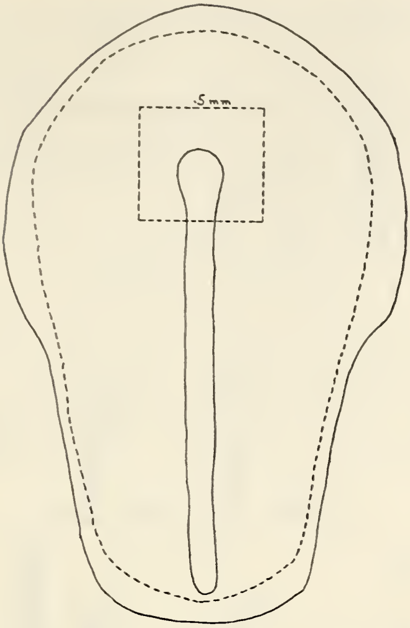
In several experiments homologous (chicken) serum as well as plasma-embryo extract exudates (*3*) were used. Several samples of commercial chicken serum were used, but proved uniformly unsatisfactory. Our own chicken serum gave good results which were essentially the same as those obtained with horse serum.

This study is based on 778 individual cultures, about three quarters of which were sectioned. In addition, 59 grafts were recovered from a total of 156 (*see* later). All these were sectioned and studied histologically.

RESULTS

Whole DPS Blastoderms

Reaggregates of whole DPS blastoderms (text-fig. 1), which were kept on a glass surface, quickly adhered to the glass and began to



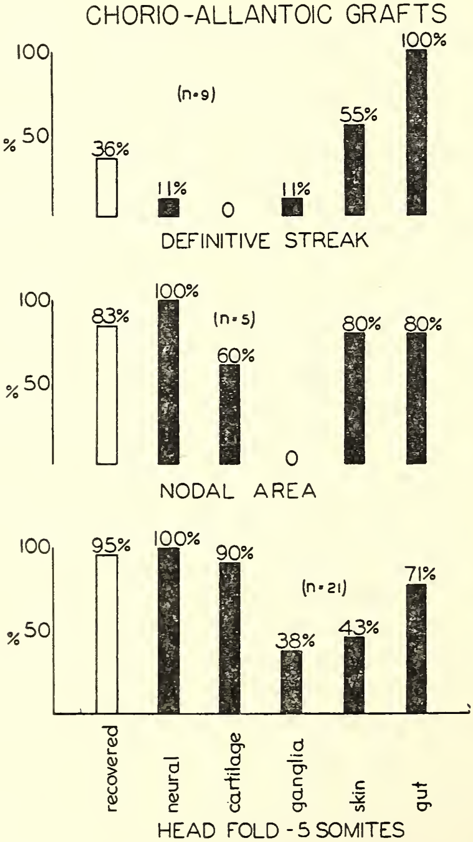
TEXT-FIGURE 1.—Diagram of definitive primitive streak (DPS) stage embryo. The *outer dashed* line indicates the amount of pellucid area used for whole DPS reaggregates. The *inner dashed* square indicates the extent of the nodal area used in these experiments (0.5 mm.^{2}).

spread in a thin epithelioid sheet away from the aggregate center. The spreading occurred so uniformly and consistently that subculturing of the reaggregate had to be effected within 8 hours of the dissociation, if unspread masses were to be used. Characteristically, the spreading tissue had a sharply demarked advancing border (fig. 2). When such spreading reaggregates were kept on a glass surface, the epithelioid migration continued until most of the mass of cells formed a thin layer. If several masses were in the same depression, the sheets merged to form a single film of cells with scattered thick areas, which usually demarked the original site of an aggregate.

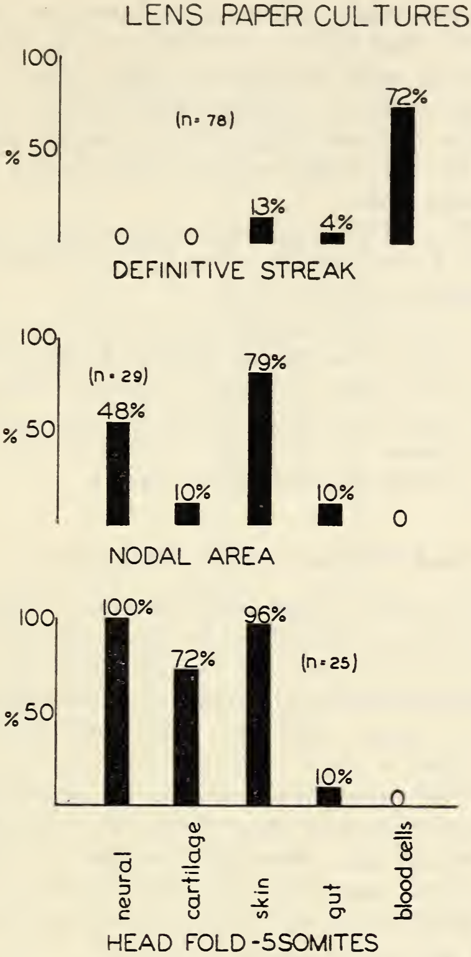
When DPS reaggregates were placed on lens paper or Millipore filter platforms, essentially the same spreading occurred. It was more marked on the Millipore filter than on the lens paper. On the latter substrate the cultures tended to grow in thickness after the initial spreading. On a plasma clot the spreading was so rapid and marked that it was difficult to subculture the tissue. When such tissues were grown on agar-impregnated lens paper, they did not spread in the characteristic way. Instead they became vesicular, or remained as fairly dense, little cultures (fig. 1). In a majority of these, concentrations of hemoglobin became evident after 3 or 4 days, but those cultured on Millipore filters were exceptions: Cultures of this sort were so thin and devoid of internal structures that it was difficult to photograph them. This type of culture has been called "simple" for convenience of description.

Chorio-allantoic or body-wall grafts of DPS reaggregates were rarely recovered (text-fig. 2). Chorio-allantoic grafts could be observed in their early stages and most of them continued to spread rapidly on the membrane surface and became dissipated, possibly merged with the membrane.

Histological examination (text-fig. 3) of the lens-paper and plasma-clot cultures revealed that they consisted, for the most part, of rather simple stromal tissues plus many cells, which I call "parenchyma," on the outer borders. A number contained smooth-muscle cells. Seventy-two percent of these cultures had well-developed erythrocytes, frequently in small endothelial-lined, sinuslike spaces (figs. 3 and 4). In a few cultures there were patches of atypical skinlike tissue (13%) as well as atypical gut (4%). In many, the parenchyma cells resembled typical yolk-sac membrane cells and were full of eosinophilic, zymogen-like



TEXT-FIGURE 2.—Bar graph depicting the incidence of certain tissues found in chorio-allantoic grafts from a representative experiment. Percent of takes indicated by *open* bars. Percents for *solid* bars indicate incidence of tissues in recovered grafts.



TEXT-FIGURE 3.—Bar graph depicting the incidence of certain tissues found in cultures from a representative experiment (*see* text).

granules. Those reaggregates that were grown on agar-coated lens-paper platforms and which invariably became vesicular were bilaminar sacs whose walls resembled early chorio-allantoic membrane (fig. 5). Occasionally the outer cells were enlarged and resembled yolk-sac cells. Characteristically these sacs were full of blood cells and in these cultures leukocytes predominated. The more solid of the cultures raised on agar-coated platforms appeared to be mostly hematopoietic tissue (fig. 6). A few of the Millipore filter cultures contained small gut vesicles, but most cultures were thin sheets 3 to 4 cells thick.

All of the few chorio-allantoic and body-wall grafts (text-fig. 2) were small and developed some gut, occasionally highly differentiated (fig. 7). This was the predominating tissue in most grafts, while skin was present in a few. There were two exceptional cases: In one there was a patch of tissue which could possibly have been neural. The best way to state this is that I am not certain that it is *not* neural. In the other case there were undoubted ganglia (but no other neural tissue) which sent nerves to nearby well-differentiated intestine. A possible explanation of these exceptions may be that these blastoderms (as indicated in the protocols) were cut closer to the streak, *i.e.*, had less pellucid area than usual.

Body-wall grafts of DPS reaggregates again typically had gut and skin. In several of these the skin was highly differentiated and had recognizable feathers or feather buds.

In summary, characteristically DPS reaggregates tended to spread and form simple cultures and grafts. The most frequently encountered tissues were blood cells, gut, and skin. Among several hundred cultures and grafts there were only two chorio-allantoic grafts that developed some neural tissue. In general, the cultures from these reaggregates tended to resemble extra-embryonic tissues.

Head Fold-to-6 Somite Reaggregates

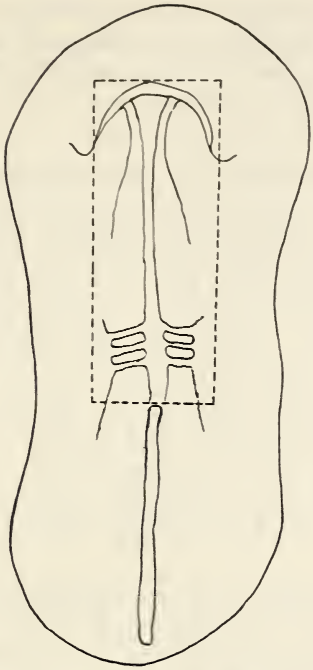
When the anterior ends of head fold-to-6 somite embryos (text-fig. 4) were subjected to the same procedure, the disaggregation and reaggregation behavior was the same as with the DPS embryos. However, the subsequent behavior was quite different. Whether they were grown on a glass surface, lens-paper platform or Millipore filter, all but a few (6%, table 1) tended to remain cohesive and massive. There was no spreading except for an occasional tongue-like outgrowth. Within 24 to 48 hours, formed organoids were evident in the interior of the cultures (fig. 8). None of these developed hemoglobin. Growth was quite rapid, but the cultures remained thick. An increase in the diameter of the culture was, for the most part, due to later growth and not to initial spreading. Such cultures are called "complex."

TABLE 1.—Summary of all cultures of DPS, nodal, and head fold-to-6 somite embryo reaggregates

	Total cultures		
	N	Complex*	Simple
DPS	189	0	189 (100%)
Nodal	36	21 (58%)	15 (42%)
Head fold-to-6 somites	191	179 (94%)	12 (6%)

*Indicates presence of neural or other tissues (*see* text).

There was considerable increase in the incidence of takes among the grafts from these reaggregates (text-fig. 2). Some of these grafts were enormous. They became vascularized quickly and grew rapidly.



TEXT-FIGURE 4.—A representative diagram for head fold-to-6 somite embryos. *Dashed* lines indicate extent of tissue used in experiment.

An analysis of the sections revealed that all but 6 percent of the cultures and all the grafts contained neural tissue. In many this was the predominating tissue (figs. 9 and 10). In some, especially those grown on agar-coated lens paper, it was the only tissue. In others, there were considerable amounts of other tissues, frequently highly differentiated. Cartilage and skin were found frequently, but gut less frequently. Occasionally skin and gut merged via transition zones (figs. 11 and 12). Some had readily recognizable smooth and skeletal muscle; a few had other tissues. None had blood cells or tissues resembling extra-embryonic membranes. The most complex cultures, veritable little teratomatous masses, developed on lens-paper platforms (fig. 9).

Grafts grown from the head fold-to-6 somite reaggregates were highly complex. They were similar to those obtained by Willier and Rawles (*12*) from chorio-allantoic grafts of whole DPS and somewhat older embryos. They frequently contained enormous blood sinuses, often in the middle of a well-differentiated structure. A complete description is superfluous since the incidence of some pertinent tissues is indicated in text-figure 2. Ganglia, not found in the cultures, were present in some of the grafts. In general the grafts were somewhat better organized than the cultures, with two of the body-wall grafts showing the most normal morphogenesis. However, no major tissue occurred in a graft which did not also appear in the cultures.

To summarize: Reaggregates from the anterior end of head fold-to-6 somite embryos behaved differently than did the DPS reaggregates (they did not spread) and characteristically formed such complex embryonic tissues as neural, cartilage, etc.

Nodal Area of DPS Embryos

The previous results led to an obvious question: Is the nodal area of a DPS embryo capable of forming complex tissues after its dissociation and reaggregation? This nodal area is well known to have a tremendous morphogenetic potential; in fact, it can give rise to virtually an entire embryo. To answer this question, experiments were undertaken in which an area of about 0.5 mm.^{2} around the node was excised from DPS embryos (text-fig. 1). Enough nodal areas (*ca.* 25) were used in a smaller volume of fluid so that the cell suspensions had about the same concentration as those used for whole DPS embryos. The usual practice was to do parallel experiments with whole DPS embryos, nodal areas, and head fold-to-6 somite embryos on the same day.

The results may be stated briefly: Approximately half (58%) the cultures from nodal-area reaggregates remained compact and massive and developed complex structures (fig. 13). Cartilage did not appear as frequently in these cultures as in cultures of the older reaggregates (text-fig. 3), but in general they were essentially similar. The rest (42%) usually spread in typical DPS fashion and remained simple. Some of these simple cultures became quite thick, even though neural tissue, etc., was absent (fig. 14). None of them developed blood cells.

Chorio-allantoic grafts of nodal-area reaggregates developed essentially as did those from the older embryos (text-fig. 2). The chief difference was in the absence of ganglia. The significance of this observation is dubious, since there are very few of these grafts.

In summary then, half the reaggregates from nodal-area cell suspensions developed complex tissues, while the other half did not.

Dilution Experiments

Since a significant proportion of nodal-area reaggregates developed complex tissue in culture while no complex tissue developed from cultures of whole DPS embryos, which of course included the nodal area, it became important to obtain information about the reason for this difference. Therefore the following three experiments (table 2) were conducted: In the first experiment, separate cell suspensions were made from nodal areas (N) and several non-nodal areas (R = the rest of the pellucid area from which the nodal area had been excised). These were then combined in different proportions (the total number of cells was kept roughly equal in each mixture) and allowed to reaggregate together and the reaggregates were followed in culture. In a second experiment, nodal areas were combined with non-nodal areas (roughly 1 non-nodal area was considered equivalent to 9 nodal areas) and these were dissociated together, thoroughly mixed, and then treated as in the first experiment. Again the total amount of tissue in each preparation was approximately the same. In experiment three, the most precise of this group, nodal areas were excised, then the non-nodal areas were cut into 0.5 mm. squares, and equal numbers of the proper combination of these equivalent squares of tissue were combined, dissociated together, and treated, from this point, as in the other experiments.

The results are summarized in table 2. As the proportion of nodal-area cells increased, the incidence of complex tissue increased markedly. In this experiment, probably because of more precise delineation of the nodal area, all the controls of nodal-area cells and most (90%) from the 3N:1R combinations developed complex tissues. A high proportion (60%) of the 1N:1R mixtures developed complex tissues. With the 1N:3R mixtures two of the experiments gave no complex cultures, while the third (#2) yielded a high proportion (12 of 14) of complex cultures. This difference may have been due to irregular cutting of the tissue or to some undetected variable in the culture situation. I regard experiment three as the most precise of the series and tend to discount experiment two somewhat. Nevertheless, if all the data are considered, the 1N:3R results fall nicely on a descending series in the incidence of complex cultures.

While these experiments make no pretense at quantitative precision, they do indicate that nodal-area cells must be present in considerable

TABLE 2.—Nodal areas combined with non-nodal areas in varying proportions

	Dilution experiment (lens-paper cultures)			
Ratio*	Experiment	N	Complex	Simple
1N : 0R	1	7	7(100%)	0
3N : 1R	1	16	13	3
	2	14	14	0
Total		30	27(90%)	3(10%)
1N : 1R	1	12	0	12
	2	16	13	3
	3	14	12	2
Total		42	25(60%)	17(40%)
1N : 3R	1	12	0	12
	2	14	12	2
	3	12	0	12
Total		38	12(31%)	26(69%)
0N : 1R	1	8	0	8
	2	6	0	6
	3	12	0	12
Total		26	0	26(100%)

*N = nodal area; R = non-nodal area (*see* text).

numbers before they can express their full morphogenetic potential. According to rough calculations based on projections against a quadrille-ruled background, the nodal area, as herein described, represents about 1/10 of the pellucid area used in these experiments. Actually the number of cells in the nodal area is probably relatively greater than 1/10, because this region is thicker than the rest of the blastoderm. Since none of the cultures from whole DPS reaggregates have developed neural tissues under the conditions of these experiments, and since the incidence of neural (complex) tissue increases with increasing concentration of nodal-area cells, then it follows that there is a minimal concentration of such cells, somewhat above the 10 percent level, required before their morphogenetic potentials may be expressed.

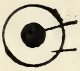
Reaggregate Combination Experiment

This experiment was prompted by an awareness of some of the possible weaknesses in those that have been described. Since there are no markers for the nodal cells and since the nodal area, in an intact DPS embryo, is notoriously the most sensitive to deleterious treatment, one may well wonder whether these data are not the result of differential mortality of the nodal cells. Relatively few cells succumb to the dissociation procedure, and the cultures, in the initial few days, are relatively free of cell degeneration. Most of the degenerative changes which I have observed occurred relatively late in the fast-growing cultures (it was most marked in plasma-clot cultures), and for the most part involved tissues that had already differentiated. Nevertheless the possibility could not be dismissed. In addition there were other questions which remained unanswered because of the absence of cell markers. Do the nodal cells sort out as did the cells in Townes and Holtfreter's experiments? Can aggregates of nodal-area cells (or others with strong morphogenetic capacity) maintain themselves against a strong opposing tendency? Direct answers are still not available, but some inferences may be drawn from the following:

Separate cell suspensions were made from whole DPS blastoderms and from the anterior halves of head fold-to-6 somite embryos. These suspensions were allowed to reaggregate separately. After reaggregation was completed, a fragment of the older reaggregate (O) was surgically inserted into a somewhat larger piece of DPS reaggregate. The size of the pieces was noted. The combined tissue was then grown on culture platforms. Controls from the original reaggregates were grown on the same platforms.

The results reveal (table 3) that even some of the relatively large fragments (0.2–0.3) of the O reaggregates failed to express their morphogenetic properties when they were placed in intimate contact with rapidly spreading tissue. Apparently they were caught in this spreading activity and were forced to spread along with the DPS tissue. The change in behavioral pattern resulted in a change of morphological expression. Nineteen percent of these larger masses failed to develop complex tissues, while none of the controls for this size class failed to do so.

Table 3.—Reaggregates of head fold-to-6 somite embryos surgically incorporated into reaggregates of DPS embryos*

Lens-paper + Millipore filter platform				
 O = hf to 6 somite reaggregates Whole DPS reaggregates		N	Complex	Simple
O in DPS				
	O = 0.1–0.2 mm.	95	69 (77%)	26 (23%)
	O = 0.2–0.3 mm.	37	30 (81%)	7 (19%)
O Controls				
	O = 0.1–0.2 mm.	80	71 (89%)	9 (11%)
	O = 0.2–0.3 mm.	20	20 (100%)	0
DPS Controls				
	0.3–0.5 mm.	64	0	64 (100%)

*See text.

When the size of the fragment of O reaggregate was decreased, a somewhat higher proportion of the cultures did not develop complex tissue. Even more significant is the fact that some (11%) of the controls for this size class developed into the simple type of culture. Supplementary experiments revealed that, with very small pieces at or slightly below the 0.1 mm. level, the nature of the substrate was important. When such small pieces were cultured on a glass surface (in fluid medium) they tended to spread and remained simple on subsequent transfer to a platform culture. Fewer evinced this tendency when placed on a platform from the start.

DISCUSSION

The experiments described lead to the inference that the early chick embryo contains two basic categories of tissues. These may be distinguished by their behavioral properties. One class is essentially extra-embryonic and is distinguished by its propensity for active spreading. Blood cells and skinlike and gutlike structures are the chief types of differentiated tissues which appear after the development of such reaggregates. The other category may be called "embryonic." Reaggregates of embryonic tissue tend to retain a massive configuration, resist spreading, and form such complex tissues as neural, cartilage, etc. In general the organization and degree of development of the tissues, *i.e.*, skin and gut, which develop from such reaggregates are more typical than those that form from the extra-embryonic types.

These two behavioral patterns appear to be opposing tendencies. The relatively smaller population of embryonic cells must be present in a considerable local majority in order to express embryonic properties. This is revealed by the dilution and combination experiments. In an intact embryo the nodal area [or Spratt's (*19*) "neural plate" area] is undoubtedly an island of embryonic cells, which is maintaining itself against the opposing tendencies of the surrounding extra-embryonic tissues. Under certain pathological conditions that lead to the formation of so-called anidian blastoderms (*20–22*), we may presume that the embryonic cells are too diluted to express their embryonic properties. In fact, Harrison speaks about the "dispersal" of the primitive streak in his discussion of the experimental production of the anidian condition. Typically, such blastoderms consist of a wide area of blood islands which may or may not have a small knot of distorted embryonic tissue in the center.

Data from various sources have indicated that the achievement of a critical mass of a group of cells with common properties may be a requisite for differentiation of some tissues. There has been indirect evidence for this from the studies of mutant syndromes, such as the *souris luxée* of Hovelacque and Noel (*23*), luxate mice (*24*), and congenital hydrocephalus of mice (*25*). In all these, precartilaginous condensation of mesenchyme fails to occur to the normal extent and, instead of cartilage, fibrous tissue develops. Similar evidence comes from the experiments of Weiss and Andres (*4*). For example, neural tissue was not present in smaller grafts (*26*). His observations led Andres to state, "Rather must we assume that there is a critical lower limit of amount of material for each organ below which it cannot express itself." More direct evidence comes from the beautiful experiments of Moscona (*6*). Studies of heterotypic mixtures of dissociated mouse and chick-embryo cells revealed that there is a liminal number of cells below which they fail to express their histiotypic properties. This may vary for different cell types and must be defined for each cell type and for each set of conditions. From this point of view it is quite interesting that the liminal values here described for the reaggregates of embryonic cells (*ca.* 0.1 mm. in diameter) are almost the same as the value found in the experiments of Grobstein and Zwilling (*27*), done under completely different conditions. The small reaggregates of older tissue, incorporated into the DPS reaggregates, were part of a large mass of tissue which was not quite as exposed to the leeching effects of a large volume of fluid as were the tissues in the 1953 experiment. Nevertheless, the liminal value for neural-tissue formation is so similar in the two experiments that it must be considered more than a coincidence.

The lower incidence of complex cultures which were obtained from nodal-area reaggregates, when compared with the older embryos, may not be due solely to the difference in concentration of embryonic cells. It may also reflect a possible difference in cell stability. Presumably the propensity for formation of complex tissues has been recently acquired, via some inductive stimulus, by the nodal-area cells. They also have metabolic properties which are different than the surrounding tissues (*28*, *29*). The realization of the embryonic properties after dissociation probably depends on the re-establishment of the proper supracellular organization and metabolic milieu—apparently less readily attained by the younger tissue than that from older embryos. The recent experiments of Denis (*30*, *31*) substantiate the point of view that a newly acquired capacity to form neural tissue is initially labile. Pieces from the animal pole of young amphibian (*Pleurodeles*) gastrulae were subjected to the influence of the archenteric roof of neurulae for short intervals (3 hours) and then removed to another site. Neural tissue formed from these explants far more frequently in the blastocoele of a host embryo than on the flank, where they were forced to participate in the *stretching* movements typical of that region.

There has been considerable concern with the important problems of the nature of the inductive stimuli for differentiation. This needs no documentation. However, it is becoming increasingly important that we learn more about the properties of cells both before and after they have been subjected to such stimuli and to the conditions under which they may express differentiative capacities. This was emphasized by Holtfreter (*32*) when he discussed the importance of the "reacting system." It has recently been emphasized again, in a dramatic way, by the experiments of Barth and Barth (*33*) which show that cells from bits of frog gastrulae may differentiate into neurones, striated muscle, etc., in an unconditioned medium which has been fortified with serum globulin. My own experiments, together with those done earlier with Grobstein, indicate that cells of a common embryonic type must be present in sufficient mass which must be maintained against the activity of other, opposing tendencies, before they may express their developmental capabilities.

The title of this symposium is "Some Problems of Normal and Abnormal Differentiation and Development." If in no other respect, I hope that I have succeeded in indicating that there is still an enormous number of problems involved in relating cell and tissue behavior to differentiation. The surface has merely been scratched and much remains to be done.

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PLATE 3

FIGURE 1.—Living DPS culture grown on agar-coated lens-paper platform. *Note* vesicular structure.

FIGURE 2.—Living DPS culture after approximately 24 hours. Grown on glass surface. *Note* epithelioid type of spreading.

FIGURE 3.—Section through DPS culture grown on lens-paper platform. *Note* blood cells (erythrocytes) and skinlike epithelium.

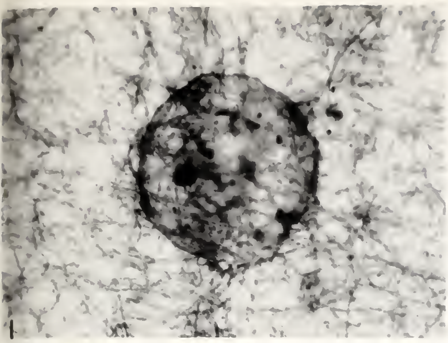
FIGURE 4.—High-power photograph of erythrocytes in DPS culture grown on lens paper.

FIGURE 5.—Section through DPS culture showing vesicular growth on agar-coated lens-paper platform. *Note* bilaminar structure reminiscent of chorio-allantoic membrane. Loose cells are predominantly leukocytes.

FIGURE 6.—Section through compact DPS culture grown on agar-coated lens paper. Central mass is unidentified glandular tissue. Most of remainder consists of hematopoietic tissue.

FIGURE 7.—Section through chorio-allantoic graft of DPS reaggregate showing well-differentiated intestine.

FIGURE 8.—Fixed and stained culture of head fold-to-6 somite reaggregate grown on lens-paper platform. *Note* formed organoids.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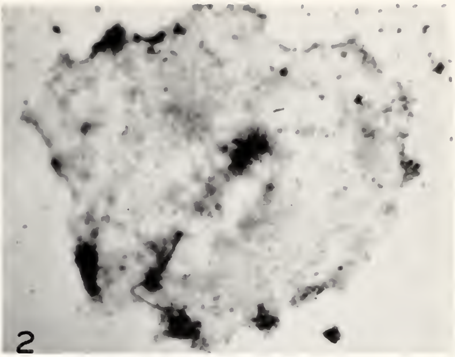
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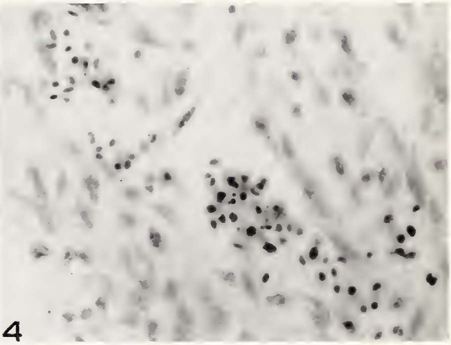
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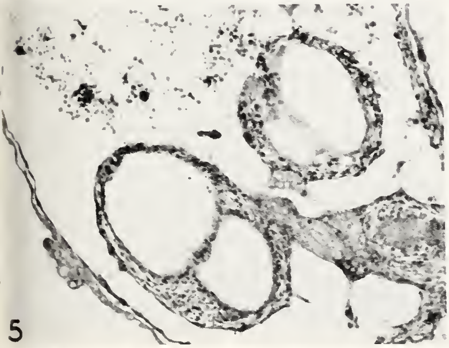
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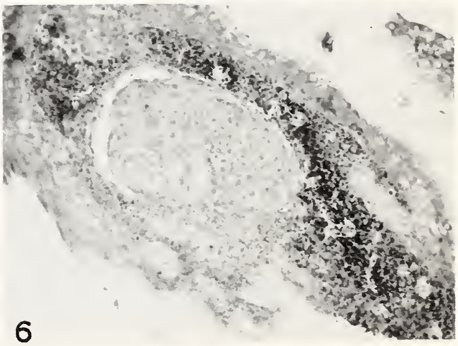
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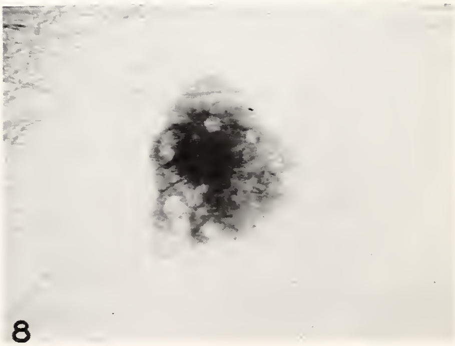
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PLATE 4

FIGURE 9.—Section through head fold-to-6 somite culture grown on lens-paper platform. Neural tissue, cartilage, and skin are present.

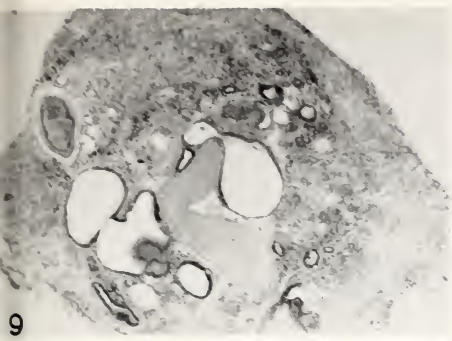
FIGURE 10.—Section through head fold-to-6 somite culture grown on Millipore filter platform. Tissue is predominantly neural.

FIGURE 11.—Section through nodal reaggregate grown on lens-paper platform. *Note* cartilage, intestine, and skin. Transition zone between skin and gut epithelium is shown.

FIGURE 12.—Higher-power view of transition zone of figure 11.

FIGURE 13.—Nodal reaggregate after growth on agar-coated lens paper. Predominantly neural. Some gut is present as is a mass of liver tissue on *right*.

FIGURE 14.—Nodal reaggregate grown on lens paper. This culture was "simple" in that neural tissue and cartilage were absent. However, culture is quite thick and consists mostly of stromal tissue, some smooth muscle, and a small amount of gut.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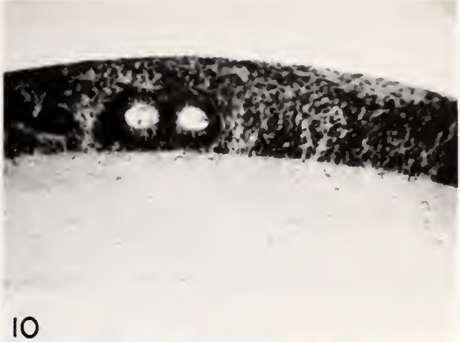
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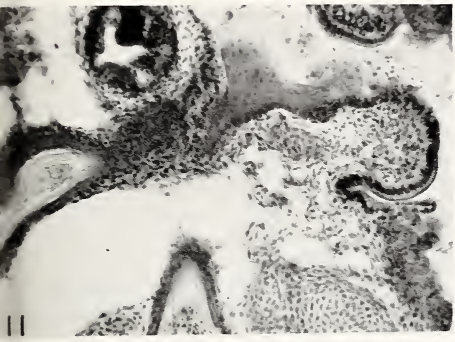
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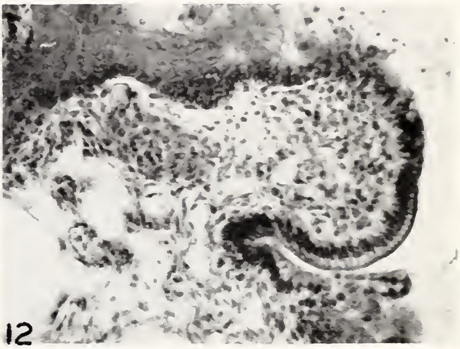
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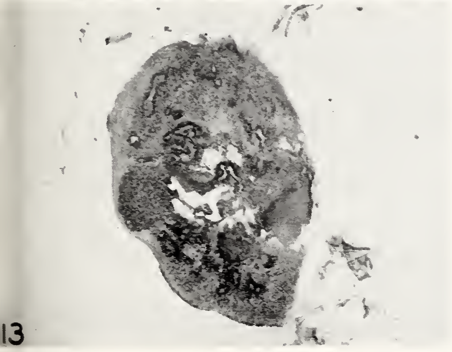
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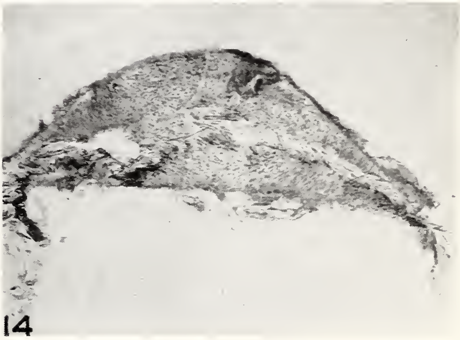
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Metabolic Mechanisms of Teratogenic Agents During Morphogenesis[1,2]

MEREDITH N. RUNNER *and* CHARLES P. DAGG,[3]
Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine

CONGENITAL deformity occurs with significant frequency in man and inbred mice. Mammalian embryos develop inside a homeostatically controlled maternal organism that tends to buffer the embryo's private pond from the rigors of external environment. Nevertheless, this sheltered form of development does not eliminate the risk that a mammalian embryo will be born with a deformity. Studies on human beings, in which the rate of spontaneous deformity is low, are necessarily based upon the census-of-population approach. In the laboratory, however, selected treatments of pregnant animals, as reviewed by Kalter and Warkany (*1*), can provide a satisfactory frequency of deformity with which scientists can experiment.

Embryonic factors which permit experimental induction of deformity, or deviations from the normal, are numerous. Eight of the more obvious determinants of susceptibility to congenital deformity are:

A. Responsiveness of the embryo.
   1. At what stage (time) is the influence effective?
   2. What is the duration of action of the influence?
   3. Where within the embryo is the responsive site?
   4. How much recovery or repair is possible?

B. Nature of the metabolic processes involved.
   1. What are the critical reactions?
   2. What reactions are indirectly related?
   3. How complete is the alteration of any one reaction?
   4. What is the effect of increased dose or intensity?

Most teratologists will view these factors as annoying variables usually beyond the reach of satisfactory control, while the embryologist will view these items as challenging questions, the answers to which will provide information about mechanisms of normal and abnormal development. Some of these determinants have been studied as more or

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[1] Presented at a symposium on normal and abnormal differentiation and development, held in celebration of the 30th anniversary of the Roscoe B. Jackson Memorial Laboratory, June 15-17, 1959, at Bar Harbor, Maine.

[2] This investigation was supported by research grant RG-4827 from the National Institutes of Health, Public Health Service, and research contract AT(30-1)-1762 from the Division of Biology and Medicine, U.S. Atomic Energy Commission.

[3] The authors wish to acknowledge the valuable technical assistance of Charles P. Ridley and Darrold Dorr.

less isolated variables by Grobstein and Holtzer (*2*), Markert (*3*), and Zwilling (*4*). Like Duffey and Ebert (*5*) we have attempted to relate some of these variables to events in the embryo as an intact organism.

Experiments to be reported here attempt to investigate (*a*) by what mechanisms embryos are deformed following teratogenic treatments, and (*b*) what the routes of action are when the same deformity is produced by different treatments. A variety of prenatal treatments with relatively acute effects has been used, and the effects of these treatments upon the axial skeleton in the region of the thorax have been studied in detail.

METHODS

Inbred mice of strain 129/RrJ have been used throughout. Females about 6 months of age and in estrus were caged with males, and the time of mating was determined within plus or minus 3 hours by the presence of a vaginal plug.

Six different treatments were selected for a study of their comparative effects on development when administered on the 9th day of pregnancy: (1) Animals subjected to fasting were kept from 8 days 0 hours to 9 days 0 hours (throughout the 9th day of pregnancy), singly, in a cage with a wire-mesh floor, with water, but no food, available. (2) To provide one third the normal partial pressure of oxygen during the experimental period, hypoxia was maintained for 5 hours, at 8 days 15 hours until 8 days 20 hours after mating. (3) Trypan blue, 0.25 or 0.5 mg., was injected intraperitoneally at 8 days 0 hours. (4) One mg. of iodoacetate was given intraperitoneally in 2 divided doses, at 8 days 0 hours and at 8 days 15 hours. (5) Ten or 15 mg. of the folic acid antagonist, 9-methylfolic acid, was given orally at 8 days 15 hours. In order to preclude extrinsic sources of folic acid during the 9th day, the mice were fasted and received orally 25 mg. of sulfasuccidine at 8 days 0 hours and 400 mg. of glucose at 8 days 15 hours. (6) The dose of radiation was 200 or 100 r; both doses were given in a single exposure at 9 days 0 hours. The dose rate at a target-specimen distance of 56 cm. was about 60 r per minute. The X rays were delivered by a 250 kvp machine using a current of 20 ma., with added filters of 0.5 mm. Cu and 1.0 mm. Al. The mice were irradiated dorsal side up, inside plastic holders on a turntable rotating at 7 rpm. The plastic chambers were inclined at an angle on the turntable to assure that every point on the axis of the animal was equidistant from the target of the X-ray tube.

The last 5 treatments, in addition to being used singly, were also combined with the first treatment listed, *i.e.*, fasting. It was anticipated that these data would enable a study of interaction between the various treatments and fasting.

The females were killed on the 18th day of pregnancy, and the fetuses were removed. After gross inspection under a dissecting microscope, the fetuses were cleared, stained with alizarinsulfonate, and examined for skeletal deformities.

RESULTS

Table 1 summarizes the experimental findings and shows the number of animals studied, the percent of fetuses with abnormal thoracic vertebral elements, and the mean number of young per litter.

Examination of alizarin-stained fetal skeletons limits observations to the ossified portions of the skeletons, though the remainder of the structures are represented as cartilage. Abnormal vertebral elements included missing neural arches, centra or ribs, or fusions of adjacent ossified structures.

Mean sizes of litters are given in table 1 to indicate the relation between prenatal treatment and possible depletion of embryos in the litters. Averages between 4.8 and 6.1 live young per litter are within the normal range for this strain of experimental animal. These observations indicate the possibility that most treatments had not been sufficiently severe to cause prenatal mortality within the litters. The right half of the table further substantiates this point because litter sizes remained within normal ranges, though 2 teratogenic treatments were concurrently effective. The average number of young per litter, 4.4 and 3.6, seen in the mothers that were X-rayed with 200 r, however, indicated that this treatment had actually killed a portion of the litters. Surprisingly enough, fasting, which caused 17.2 percent loss of maternal body weight within 24 hours (table 2), failed to bring the first 4 treatments up to the lethal level. Except for the animals X-rayed at 200 r, death of the fetuses is neither correlated with nor causally associated with the frequency of induced deformity.

TABLE 1.—Comparison of frequencies of fetuses with defective vertebral elements after treatments that induce congenital deformity

	Food available *ad libitum*			Fasted 24 hours throughout the 9th day post coitum		
Treatment during 9th day	Number of fetuses	Percent abnormal	Size of litters	Number of fetuses	Percent abnormal	Size of litters
None	49*	0	5. 4	57	24. 0	5. 2
Hypoxia, 5 hours at 7 percent	131	47. 3	5. 7	73	75. 3	4. 9
Trypan blue, 0.5 mg.	59	16. 9	5. 4	115	43. 5	5. 5
Iodoacetate, 1.0 mg.	37	62. 6	5. 3	62	66. 1	5. 6
9-Methylfolic acid, 10–15 mg.	128*	13. 3	6. 1	178	18. 5	5. 7
X ray, 200 r	44	100. 0	3. 6	83	94. 0	4. 4
X ray, 100 r	46	13. 0	6. 6	43	32. 5	4. 8

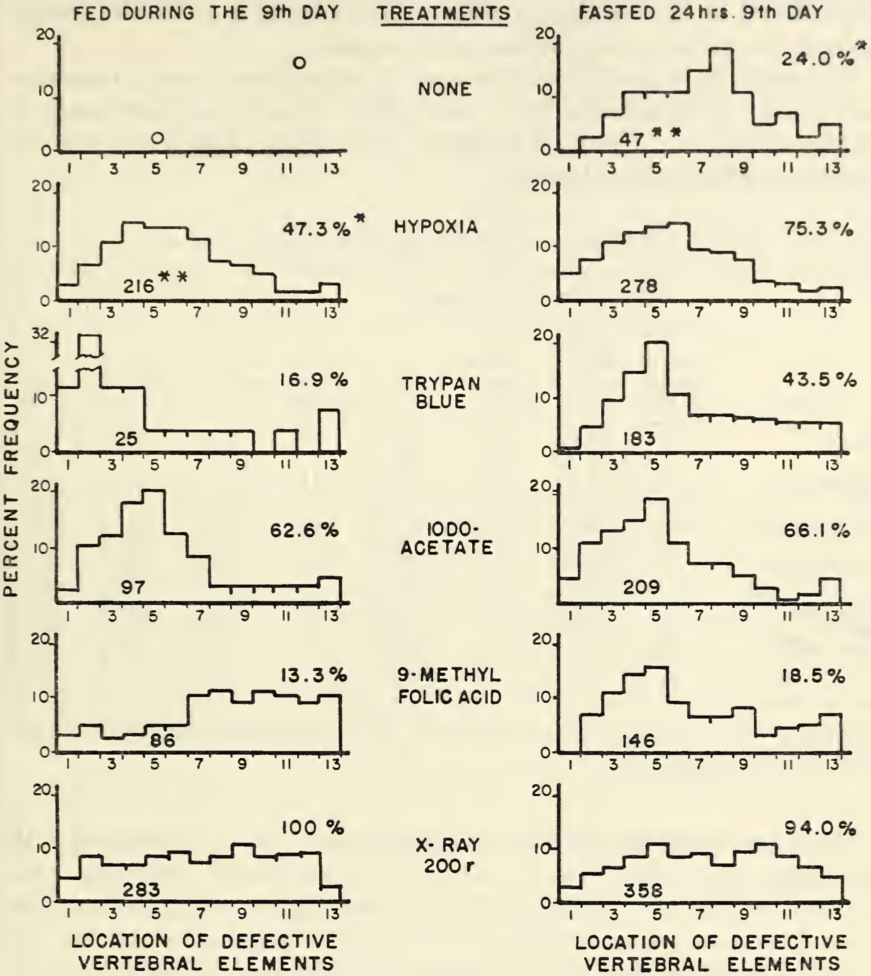
*These animals differed insofar as they were deprived of food throughout the 9th day, but protected from the fasting effect by 400 mg. of glucose given by stomach tube 8¾ days post coitum.

The percentages of fetuses with abnormal thoracic vertebral elements ranged from 0 among the fasted but glucose-protected animals, to 100 percent among the animals given 200 r of X radiation. The differences between frequencies of abnormalities among the groups receiving single treatments have little significance other than to indicate the relative intensity of the treatments. Should the need arise, severity of these treatments could be adjusted to give more uniform frequencies of deformity.

When the frequencies of anomalies after single and dual treatments are compared, the data become more meaningful. Three of the treatments combined with fasting are clearly nonadditive, 63 as compared to 66 percent of deformed fetuses following treatment with iodoacetate, and 13 as compared to 18 percent deformed fetuses after 9-methylfolic acid. Treatment with 100 r of X ray resulted in 13 percent of fetuses with minor deformities. The effect of combined treatment of fasting and 100 r does not significantly differ from the result of fasting alone. It appears that 100 r of X ray is nonadditive when combined with fasting. Two treatments combined with fasting are remarkably close to being additive: 47 plus 24 percent equals 71 percent and approximates the 75 percent deformed fetuses observed among the animals treated with hypoxia; 17 plus 24 percent equals 41 percent and approximates the 43 percent deformed fetuses treated with trypan blue. Results from treatments with 200 r of X ray have little significance in this connection because the incidence was maximal with 100 percent abnormal fetuses, even without subjecting the animals to fasting.

Text-figure 1 shows the sites at which defects occurred in the abnormal young. The numbers above the graphs repeat the frequencies of deformed fetuses given in table 1 for sake of reference. The numbers within the histograms show the number of defective vertebral elements observed. The left half of text-figure 1 shows the distributions of deformed ribs and thoracic vertebrae when each of 5 treatments was administered on the 9th day after mating. Obviously there is no correlation between the frequency of deformed fetuses and the frequency of defective vertebral elements. Location of defective vertebral elements must therefore correlate with something other than relative intensity of treatment.

The right half of text-figure 1 shows the observations after the mothers were fasted, and after fasting was combined with other teratogenic treatments. The frequency distributions following hypoxia, trypan blue, iodoacetate, and 9-methylfolic acid combined with fasting are remarkably similar with modes at the fifth or sixth thoracic vertebra. The similarity of this group of distributions is distinctly unlike that from single treatments, and the modes are shifted anteriorly when compared to fasting as a single treatment. These similar distributions of abnormal vertebrae existed in spite of the fact that the frequency of defective fetuses ranged from 18 to 75 percent. The modes of the distributions of defects therefore did not correlate with the relative intensity



TEXT-FIGURE 1.—Histograms depicting the sites of occurrence and percent frequencies of deformed ribs and thoracic vertebrae.

* Indicates percent of fetuses with defective vertebrae; *see* table 2.

** Indicates total number of defective vertebral elements in experimental group.

of the treatments, as indicated by the percentage of defective vertebral elements.

The frequency distributions for abnormal vertebrae after combined treatments, when compared to the distributions for single treatments, show that the mode for trypan blue was shifted posteriorly and that for 9-methylfolic acid was shifted anteriorly, whereas with hypoxia and iodoacetate the modes did not shift when fasting was superimposed. This similarity in the distribution of defects existed in spite of the observation that the percent of fetal abnormalities was additive after hypoxia and nonadditive following treatment with iodoacetate. Combined treatments, as indicated by the location of defects, have seemed to make the groups more similar in fasted than in nonfasted animals.

The mode for the effect of 200 r of X ray on the thoracic vertebrae is detectable with uncertainty, if one exists. A more complete analysis of the teratogenic effects of radiation at different dose levels will be given in a subsequent publication.

TABLE 2.—Maternal characteristics of experimental animals

Treatment	Food available *ad libitum*				Food deprived 24 hours			
	Number of mothers	Weight at conception (*gm.*)	Loss of weight (*percent*)	Age (*days*)	Number of mothers	Weight at conception (*gm.*)	Loss of weight (*percent*)	Age (*days*)
None	9*	27. 0 ±1. 3†	15. 2 ±2. 3	147. 6 ±38. 7	11	28. 3 ±1. 5	17. 2 ±1. 8	159. 6 ±19. 8
Hypoxia	26	27. 9 ±2. 1	8. 2 ±3. 19	180. 8 ±48. 6	15	28. 7 ±3. 2	16. 2 ±1. 9	188. 1 ±68. 7
Trypan blue	11	28. 5 ±1. 8	0. 85 ±1. 7	208. 0 ±72. 4	21	28. 5 ±2. 3	16. 1 ±2. 4	193. 2 ±48. 5
Iodoacetate	7	28. 9 ±2. 7	9. 6 ±2. 4	204. 1 ±66. 3	11	28. 0 ±1. 2	13. 9 ±2. 0	176. 6 ±40. 8
9-Methylfolic acid	20*	27. 9 ±1. 8	14. 8 ±4. 1	191. 3 ±52. 9	31	27. 8 ±2. 6	15. 6 ±2. 5	185. 1 ±49. 5
X ray, 200 r	12	—	—	171. 3 ±43. 1	19	24. 7 ±1. 8	18. 2 ±3. 3	195. 9 ±47. 0

*These mothers were deprived of food for 24 hours and the young were protected by feeding 400 mg. of glucose by stomach tube.

†± = Standard deviation.

Since the location of defective vertebral elements was correlated with something other than intensity of treatment, maternal influences, with litters as units, were studied. Maternal weights at conception, percent loss of maternal weight during the 9th day of pregnancy, and the age of the mothers at time of treatment are summarized in table 2. Each fetus was assigned a score according to the number of defective thoracic vertebral elements. This score was transformed by the method of Freeman and Tukey (*6*), $X = \sqrt{x} + \sqrt{x+1}$, and the mean transformed score for the litter represented the response of the mother, or litter as a unit, to the experimental treatments. The transformed scores for litters permitted correlations to be calculated for relationship between the weight of the mother at conception, percent loss of weight during the 9th day, and age at time of treatment. The correlation coefficients are shown in table 3. Age of the mother at treatment varied considerably (3 of the standard deviations extended more than 2 months on each side of the mean), but showed no correlation with the mean litter score in the 11 samples of animals. Age of the mother within the ranges studied, approximately 4 to 9 months, was found not to be correlated with magnitude of response of the litters.

TABLE 3.—Correlation coefficients testing possible relationships between transformed litter score* and maternal characteristics

	Food available *ad libitum*				Food deprived 24 hours			
Treatment	Number of mothers	Weight at conception (*gm.*)	Loss of weight (*percent*)	Age (*days*)	Number of mothers	Weight at conception (*gm.*)	Loss of weight (*percent*)	Age (*days*)
None	9	−0.230	+0.639†	−0.391	11	−0.459	−0.842‡	+0.196
Hypoxia	26	+0.191	+0.474†	+0.203	15	+0.468	+0.006	+0.388
Trypan blue	11	+0.560†	+0.602†	+0.446	21	−0.064	+0.063	+0.143
Iodoacetate	7	−0.228	+0.450	+0.219	11	+0.410	−0.386	+0.458
9-Methylfolic acid	20	+0.558†	−0.155	−0.172	31	−0.182	−0.358‡	+0.168
X ray, 200 r	12	—	—	+0.528	19	−0.178	−0.178	−0.200

*Method of Freeman and Tukey (*6*).

† = $P<0.05$.

‡ = $P<0.01$.

Weight of the mothers at conception showed a significant correlation with the transformed litter score in 2 of the 11 samples of litters, in mothers treated with trypan blue and in those treated with 9-methylfolic acid, with food available. The correlated response was inconsistent insofar as these same treatments produced no significant correlation when food was withheld. It is concluded that there were no consistent correlations between weight of the mother at conception and magnitude of response of the litter units.

Loss of maternal weight during the period of treatment, the 9th day of gestation, showed significant correlation coefficients in 5 of the 11 samples of litters. There is no obvious pattern to the distribution of these correlations and no consistency between the significant correlation and treatments. Therefore the data may be interpreted to suggest but not to demonstrate a relationship between percent weight loss of the mother and magnitude of response of the litter.

In addition to the vertebral anomalies, each of the treatments produced a variety of other skeletal malformations. Cranioschisis was a common finding, at a low percentage, in all groups. The complete array of responses produced by each treatment, with the omission of vertebral and cranial malformations, are reviewed in table 4. Tail abnormalities, shortening and various forms of bending, were observed after treatments with trypan blue and 200 r of X ray. Abnormal neural arches of cervical vertebrae were observed in fasted mothers in which treatments had been combined with iodoacetate and 9-methylfolic acid. Defective centra of lumbar vertebrae, except for fasted animals and controls, occurred following all treatments which were combined with fasting. Posterior shifting of the costal border, *i.e.*, 14 pairs of ribs, was a consistent finding for 4 of the treatments, both with and without fasting. All the side effects were present in relatively low frequencies, but were well above the frequencies seen among spontaneous, sporadic defects. Thus, considering the total array of defects, each treatment showed a characteristic syndrome of deformations.

TABLE 4.—Comparison of characteristic syndromes of various treatments

	Animals fed				Animals fasted			
Treatment	Tail	Cervical vertebrae	Lumbar vertebrae	14 Pairs of ribs	Tail	Cervical vertebrae	Lumbar vertebrae	14 Pairs of ribs
None	—	—	—	—	—	—	—	—
Hypoxia	—	—	—	X	—	—	X	X
Trypan blue	X	—	X	X	X	—	X	X
Iodoacetate	—	—	—	X	—	X	X	X
9-Methylfolic acid	—	—	X	—	—	—	X	—
X ray, 200 r	X	—	X	X	X	X	X	X

DISCUSSION

A general discussion of these results will attempt to indicate how analyses of tissue metabolism and tissue interactions can be correlated with studies on teratogenic treatments of mammalian embryos.

Identical end results of different teratogenic treatments would be expected to be relatively rare, since a number of the determinants of congenital deformity listed on page 41 cannot be equilibrated. Murakami (*7*) has attributed malclosure and hyperplasia of the neural tube as common effects from 6 teratogenic procedures. Cleft palate as a definitive morphological entity has been produced by many teratogenic treatments including X ray, cortisone, folic acid inhibition, anoxia, and vitamin E deficiency. The potential danger of accepting superficial resemblance as true "chemocopy" of teratogenic agents has been demonstrated by Warkany (*8*). Undoubtedly the most intensive comparison of similar response to different teratogenic procedures has been analyzed by Landauer (*9*). He showed that two agents, insulin and pilocarpine, gave similar limb defects and both were protected by nicotinamide, but the key finding was that there were quantitative differences in the different portions of the limb, *i.e.*, femur, tibiotarsus, and tarsometatarsus, depending upon the agent employed. In order to approximate similar end results, one must empirically arrive at comparable effective intensities and durations of influence. Time of initiation, rate of action, and cessation of action of agent cannot be precisely controlled; therefore different agents may influence quite different processes in rapidly changing embryos whose end results are being compared. On the basis of these considerations, the methods of timing and administering the teratogenic treatments for this report were varied, depending upon estimated metabolic effects of the treatments employed. The degree of success can be judged by the similar patterns of location of defective vertebral elements, text-figure 1, and the relatively few types of "side effects" observable in skeletal preparations (table 4).

Although all 6 treatments altered the morphogenesis of thoracic vertebrae and ribs, each treatment showed differing degrees of individuality. These side effects, often attributed to specificity of agent, may be the consequence of a strikingly different balance of some, or all, of the determinants of susceptibility to deformity listed on page 41. There is no *a priori* basis and little gainful purpose for speculating upon the mechanisms of agent specificity. The consistent observations, on the other hand, that all treatments had by far their most pronounced effect on the thoracic vertebrae are not only remarkable but, perhaps more significant, have suggested a common route of action for the various treatments. It appears valid to accept the proposition that varied stimuli can result in the same response through a common metabolic pathway.

The array of treatments causing deformed vertebrae has suggested that a common feature at the metabolic level is that they interfere with energy production from carbohydrate oxidation (*10*). The metabolic scheme involving glucose and oxygen together with enzymatic factors, recently advanced by Runner (*11*), needs further proof.

The quite different approaches for the study of descriptive morphogenesis and the metabolic bases for morphogenesis have resulted in divergent points of view. However, current trends in embryology unite the descriptive and metabolic approaches to causal embryology. Considering the problem of vertebral development from the descriptive point of view, one is impressed by the close association between neural tube, notochord, and somites (*12*).

Grobstein and Holtzer (*2*) have shown the neural tube to be an inductor of cartilage from somitic sclerotome. Both inductor neural tube and liminal amounts of competent mesoderm are prerequisites to chondrogenesis. Effects of subcritical masses of precartilagenous condensations of mesenchyme have been reported by Carter (*13*) and Gruneberg (*14*) to be associated with the genetic deformities, luxate, and hydrocephalus in mice. The processes involved in sculpturing the cartilage are more intricate and the activity of the notochord has been implicated. In the experiments presented here, the time of treatment of the mother coincides with the first appearance of the somites of the trunk region in the embryo. Subsequent to the treatments the somites form in their normal arrangement, as nearly as can be judged by gross examination of the embryos. It would be important for us to understand what occurs within the embryo in the nature of the tissue interactions, such as Markert (*3*) and Zwilling (*4*) have described for isolated tissues.

A phenomenon that one sees not infrequently when working with experiments correlating congenital deformity and morphogenesis may be called the "ambush effect." At the time the X-ray machine was turned off, the embryos under investigation were approximately of 20 somites. Neither the rear limbs, tail, nor their associated somites had visibly differentiated, yet a significant number of the young appeared with malformed tails. In other words, the treatment with X ray had so altered the embryo that, when the tail appeared several morphological stages later, development was abnormal. This phenomenon is not a new one (*15*), and is in fact suggestive of differential gene action for the time of appearance of enzyme or substrate, proposed by Markert (*16*). Treatments may for an indefinite time reduce levels of sensitive enzymes or substrates which, many developmental stages later, become critical components for morphogenesis. This set of circumstances would explain the "ambush effect" and would account for the appreciable delay between the primary effect of treatment and abnormal morphogenesis.

Table 5 shows how the interaction of fasting with the other 5 treatments can be characterized as an interfering, a nonadditive, an additive, or a reinforcing effect.

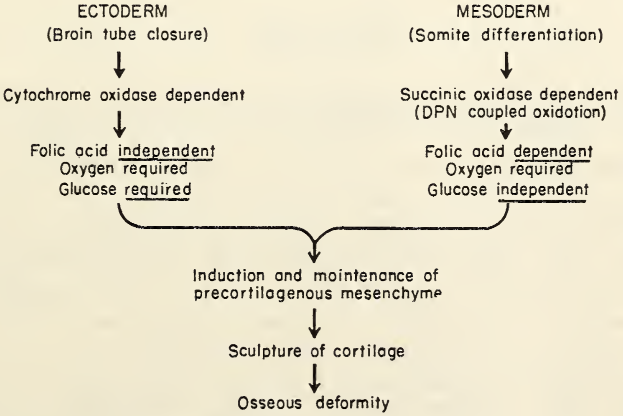
TABLE 5.—Possible patterns of interaction between different teratogenic treatments

Possible effects of combined teratogenic treatments	Examples from treatments combined with fasting
Interference effect Combined effects are less than those produced by the single treatment having the larger effect.	
Nonadditive effect Combined effects are approximately those produced by the single treatment having the larger effect.	Iodoacetate 9-Methylfolic acid X ray, 100 r
Additive effect Combined effects are approximately the sum of the individual effects.	Hypoxia Trypan blue
Reinforcing effect Combined effects exceed the sum of the individual effects.	Insulin*

*Preliminary interpretation from unpublished data.

Combinations of teratogenic treatments have also been used on several occasions in an effort to search for additive or reinforcing influences from subteratogenic levels of various treatments. Kalter (*17*) reported that the incidence of cleft palate was increased by fasting the mice for 72 hours and giving them subteratogenic doses of cortisone. Miller (*18*) found that fasting C57BL mice on the 9th day gave 1 percent of fetuses with cleft palate, and cortisone administered on 11, 12, and 13 days gave 24 percent of fetuses with the same defect. When both treatments were given to the same animals, the frequency of abnormal palates was 76 percent. Although the data from Kalter's observations are not available, those of Miller showed a reinforcing effect. Smithberg (unpublished data) has found that the teratogenic effects of insulin in mice are increased by the addition of nicotinamide to the experimental treatment. Woollam and his colleagues showed that cortisone and thiouracil potentiate the teratogenic effect of hypervitaminosis A (*19*), and that cortisone potentiates the teratogenic effect of X ray (*20*). These observations, as well as the results reported in this paper, are not to be compared with attempts at protective effects or alteration of stress factors, diet, etc., that in themselves are not known to be teratogenic. The present report differs from previous studies insofar as combined treatments showed nonadditive and simple additive effects but failed to find reinforcing effects.

Interpretation of the mechanism of action of the additive and nonadditive effects of teratogenic treatments can be drawn by analogy from suggestions made by Duffey and Ebert (*5*). Their conclusions can be put into a model that can apply to the problem of teratogenesis, as seen in text-figure 2. Close association of early embryonic ectoderm, neural tube, and somites is necessary for the differentiation of vertebrae and ribs, discussed by Runner (*12*). Duffey and Ebert (*5*) by using inhibitors, cyanide and antimycin A, on the chick blastoderm showed that brain was sensitive to the former but not to the latter, whereas mesoderm showed reversed sensitivity. Considering the metabolic inhibitions of the 2 treatments, it was suggested that neural tissue was critically dependent upon cytochrome oxidase, and mesoderm was differentially dependent upon succinic oxidase. Since interaction of both neural tube and somites is essential for normal morphogenesis of the vertebrae and ribs, inhibition of one or both of these oxidative pathways would be expected to lead to deformity [*see* scheme for teratogenic actions on 2 essential routes, Runner (*10*)]. The teratogenic experiments reported herein and the enzymatic dependency indicated by Duffey and Ebert suggest the interpretation given in text-figure 2 that somite differentiation (succinic oxidase activity) is dependent upon the presence of a folic acid cofactor, and that neural tube metabolism


ECTODERM
(Brain tube closure)
Cytochrome oxidase dependent
Folic acid independent
Oxygen required
Glucose required
MESODERM
(Somite differentiation)
Succinic oxidase dependent
(DPN coupled oxidation)
Folic acid dependent
Oxygen required
Glucose independent
Induction and maintenance of
precartilagenous mesenchyme
Sculpture of cartilage
Osseous deformity

TEXT-FIGURE 2.—Scheme to indicate the theoretical modes of action of treatments that produce congenital deformity.

(cytochrome oxidase activity) is dependent upon the presence of glucose. Both components require the presence of oxygen. Specific applicability of this interpretation is that each teratogenic treatment employed can fit the model of substrate-enzyme dependency, as shown in table 6, in which each single and dual treatment experiment is summarized and interpreted in terms of possible mode of action. The general applicability of the interpretations is that different tissues within the embryo possess particular complements of enzyme systems for accomplishing energy exchange. Substrate and cofactor requirements differ, and thereby render tissues differentially susceptible to depletions and inhibitions of key substances.

Table 6.—Possible interpretation of metabolic effects of teratogenic treatments combined with fasting

	Animals fed		Animals fasted		
Treatment	Abnormal fetuses (*percent*)	Possible action	Abnormal fetuses (*percent*)	Combined effect	Interpretation
None	0	Control	24	—	Decrease glucose substrate
Hypoxia	47	Reduces oxygen substrate	75	Additive	Decrease 2 substrates, glucose and oxygen
Trypan blue	17	Reduces placental transfer (oxygen?)	44	Additive	Decrease substrates (*cf.* hypoxia)
Iodoacetate	63	Inhibits glycolysis	66	Nonadditive	Glycolysis blocked and glucose substrate decreased
9-Methylfolic acid	13	Inhibits DPN coupled oxidation	19	Nonadditive	Oxidation blocked and glucose substrate decreased
X ray	100	Decreases enzymatic activity	94	—	

SUMMARY

Six different teratogenic treatments were selected for a study of their comparative effects on the morphogenesis of the thoracic vertebral elements of inbred mice of strain 129/RrJ. Fasting, hypoxia, trypan blue, iodoacetate, 9-methylfolic acid, and X irradiation were administered during the 9th day post coitum. The last 5 treatments, in addition to being used singly, were also combined with the first treatment, *i.e.*, fasting. Each of the procedures produced abnormalities in the vertebral elements, including such deformities as missing neural arches, centra, and ribs, or fusions of adjacent ossified structures.

When combined with 24 hours of fasting, some treatments (hypoxia and trypan blue) showed additive effects, and others (iodoacetate, 9-methylfolic acid, and 100 r X radiation) showed nonadditive effects on the frequency of vertebral deformities. The frequency of distribu-

tion of defective vertebral elements within the embryos showed an individuality of effects for single treatments. However, when combined with fasting, this individuality was reduced or absent.

Correlations between scores for whole litters and three maternal parameters, age at conception, loss of body weight during treatment, and weight at conception, showed no consistent pattern associated with treatment. No causative association can be made, therefore, between response of litters and the three maternal characteristics.

Interpretation of the common effects of teratogenic agents and metabolism of ectoderm and mesoderm concerned with morphogenesis of skeleton is discussed in terms of enzyme and substrate associated with oxidative metabolism.

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Session II. Immunology

Chairman: George D. Snell,
Roscoe B. Jackson Memorial
Laboratory

Speakers:

Leonhard Korngold. *Tissue-Specific Antigens.*

James D. Ebert and Louis E. DeLanney. *Ontogenesis of the Immune Response.*

Henry J. Winn. *The Immune Response and the Homograft Reaction.*

Tissue-Specific Antigens [1, 2]

LEONHARD KORNGOLD,[3, 4] *Division of Experimental Pathology, Sloan-Kettering Institute for Cancer Research, and Sloan-Kettering Division of Cornell University Medical College, New York, New York*

THE search for specific tissue antigens goes back to the days of Metschnikoff (*1*), but as will become apparent in the discussion, only a few antigens have been found and characterized. This search has utilized all the major immunologic techniques:

1. The study of cytotoxic effects of antiorgan serums on the animal or, more refined, on cells grown in tissue culture (*2-4*).
2. The localization of antiorgan serums *in vivo* (*5*).
3. Complement fixation (*6*).
4. Anaphylactic reactions (*7*).
5. Cell agglutination (*8*).
6. Staining with fluorescent antibody (*9–11*).
7. Precipitin reactions.
   a. Quantitative precipitin reactions (*12*, *13*).
   b. Double gel diffusion (*13–15*).

The advantages and disadvantages of these methods have recently been outlined at a symposium on tissue-specific antigens (*16*). The present paper will be concerned with the use of double gel diffusion in the analysis of tissue antigens. The method of double gel diffusions as used in our laboratory, the interpretation of the resulting data, and the problems associated with the immunologic analysis of human tissues have been described in previous reports (*17–18*).

Under the term "tissue antigen" we will include all soluble and insoluble antigens produced by individual cells, organs, and tissues. They comprise those antigens that are excreted by the cell during its lifespan and those that remain attached to, or within, the cell.

The term "specific antigen" will be used according to its immunochemical meaning: A specific antigen is a substance containing at least one antigenic configuration (antigenic determinant) that is absent from

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[1] Presented at a symposium on normal and abnormal differentiation and development, held in celebration of the 30th anniversary of the Roscoe B. Jackson Memorial Laboratory, June 15–17, 1959, at Bar Harbor, Maine.

[2] This investigation was supported by research grants C-2359 and C-4343, National Cancer Institute, National Institutes of Health, Public Health Service.

[3] *Present address:* Hospital for Special Surgery, Philip D. Wilson Research Foundation, New York, N.Y.

[4] The author wishes to thank Dr. Fred Stimpert of Parke, Davis & Company Research Laboratories and Dr. Chester M. Southam of the Sloan-Kettering Institute for supplying the human cells grown in tissue culture. The assistance of Miss Gerda van Leeuwen and Mr. Rudolph Smith is also acknowledged.

other substances. The antibody capable of reacting with this determinant is the "specific antibody"; other determinants on the same molecule may be found also in other substances, and antibodies against these determinants are "cross-reacting" antibodies. Therefore, in order to study the specificity of an antigen, two requirements must be met: (1) The antigen must have a unique determinant, and (2) the antiserum must contain antibody against this determinant.

Similarly, immunologic relationships among different antigens can be demonstrated only if the antiserum contains antibody against those determinants that the antigens have in common. Workers in the field of tissue antigens usually distinguish between: (1) species specific antigen, *i.e.*, antigens found only in one species, (2) organ specific antigens, *i.e.*, antigens that are unique for one organ, and (3) antigens found in all or most cells.

The injection of a pure protein from a given species into animals of another species may result in the formation of antibodies against several or all of the antigenic determinants. If some of these determinants are also present on other proteins from the donor species, or proteins obtained from other species, then the antiserum will react ("cross-react") also with them. Whether a substance is a "specific antigen" or not is a rather academic question, since the answer depends on the characteristics of the antiserum. For example, the injection of human serum albumin into rabbits usually results in antiserums that react only with the homologous antigen. However, antiserums against rabbit albumin, produced in goats, do cross-react with human albumin (fig. 1, cup C) as well as with albumin of other species, which indicates that the human albumin has antigenic determinants in common with the albumins of other animals. From the same figure it can also be seen that most of the antibody is specific for rabbit albumin; this is indicated by the long spurs that form when the homologous protein is compared with the heterologous albumins. These specific antibodies diffuse through the precipitate formed by the heterologous antigens and form an extremely heavy precipitate with the homologous albumin beyond the point of junction of the two lines (*19*). Moreover, the heterologous albumins differ among each other; for example, a spur is formed between human (cup C) and rat albumin (cup D), which indicates that the rat albumin lacks some of the determinants shared by the albumins of rabbit and man.

Rabbit antihuman γ-globulin serums, however, invariably cross-react strongly with γ-globulins from other species (fig. 2); most of the antigenic determinants with which these antiserums react are "species specific," *i.e.*, they react only with determinants of human γ-globulin, as indicated by the long spurs extending beyond the point of junction of the lines formed by the homologous and the cross-reacting antigen.

Serum albumin and γ-globulin represent, according to our definition, specific tissue or cellular proteins; the first is specific for liver, the second for cells of the plasma-cell family. The γ-globulins have been studied rather extensively by immunologists, and they have served as a model system for the study of the less-known intracellular antigens. It may, therefore, be pertinent to discuss their antigenic properties more extensively. The γ-globulins are a heterogeneous group of proteins, named on the basis of their electrophoretic mobilities. In the ultracentrifuge they appear as two components: The main component has a sedimentation constant of 7 Svedberg units (S); the minor component, known as the macroglobulin, has a sedimentation constant of 19S. The immunization of rabbits with γ-globulins frequently results in antiserums capable of reacting with both proteins (*20*). Such antiserums contain a mixture of antibodies; some react only with the 7S γ-globulin, others are specific for the 19S globulin, and a third group reacts with those antigenic determinants that both globulins have in common (*20*, *21*). Such antiserums produce two lines with normal human serum (fig. 3). The line closest to the antiserum cup (cup D) is formed by the 7S globulin, which combines with the antibodies that are specific for it and those antibodies that can combine with both 7S and 19S globulins. The line closest to the antigen cup (C) is formed by the 19S globulin and the antibodies specific for it. The antibody capable of combining with both proteins is always removed by the protein that is present in greater concentration, *i.e.*, the 7S globulin of normal serum. However, serums from patients with macroglobulinemia contain so much macroglobulin, and often so little 7S globulin, that the line formed by the 19S protein is ahead of the 7S globulin line (fig. 3, cup A), and under these conditions the antibody capable of combining with both proteins reacts with the 19S globulin.

In a previous paper (*21*) it was postulated that the immunization of rabbits with completely homogeneous 7S globulin might give rise to antiserums capable of cross-reacting with pure 19S globulin, since these two proteins share antigenic determinants. Such antiserums are now available. An actual comparison between the reactions obtained with such an antiserum (anti-7S globulin) and the antiserums obtained by immunizing rabbits with mixtures of 7S and 19S globulins (anti-7S, 19S globulin) is given in figures 3 and 4.

As was indicated, an antiserum against a mixture of 7S and 19S γ-globulin in cup D (fig. 3) forms two lines with normal serum in cup C. The heavy line closest to cup D is produced by the 7S globulin; the thinner line that curves toward cup C is produced by the 19S globulin. Two lines are formed because the antiserum in cup D contains antibodies that are specific both for 7S and 19S globulins. The antiserum against 7S γ-globulin (cup B) produces one line only with normal serum, since it lacks antibody that is specific for 19S globulin. Both antiserums, however, do react with the macroglobulin in the serum from a patient with macroglobulinemia. That it is the macroglobulin which reacts is apparent from the curvature of the line (*22*). Note that, when this macroglobulin reacts with anti-7S, 19S globulin serum (cup D), the line intersects the 7S line formed by normal serum and fuses with the 19S line of the normal serum. When the macroglobulin reacts with the anti-7S globulin serum, its line fuses only partially with the 7S globulin line. This can be explained by the fact that this antiserum contains antibody against groupings that the two proteins have in common as well as antibodies specific for the 7S globulin. In figure 4, γ_2-globulin was substituted for normal serum in cup C; the other reagents were the same as in figure 3. Note the "double spur" formed by 7S and 19S γ-globulins when they react with anti-7S, 19S γ-globulin, and the single spur when they react with anti-7S globulin. These experiments provide direct evidence for the antigenic relationship between the light and heavy γ-globulins, and are in agreement with findings reported elsewhere (*20*, *21*). The 19S globulin can be depolymerized to a monomer with a sedimentation constant of 7S, but this monomer is antigenically different from the 7S γ-globulin (*23*).

The examples we have presented so far have dealt with serum proteins. Thyroglobulin, or more accurately the thyroglobulins, is an extracellular colloid of the thyroid gland. These proteins, which are produced only in the thyroid, have recently received renewed attention, since they can give rise to autoprecipitins in the serums of patients with Hashimoto's struma (*13*). That thyroglobulin is really a mixture of at least two proteins with different antigenic properties is best demonstrated by such serums. Some of them will produce two distinct lines with highly purified human thyroglobulin (fig. 5, cup A), whereas others produce one line only (fig. 5, cup C). The two components of thyroglobulin, however, must also share some antigenic groupings, as both lines produced by one serum fuse with the single line formed by the others (*24*). Moreover, the thyroglobulins cause different immunologic responses, since some patients produce autoantibody with a sedimentation constant of 19S (*24*), whereas others produce autoantibody with a sedimentation constant of 7S. The intense immunologic response of such patients to these autoantigens is remarkable, because most autoimmune stimuli in animals are so slight that without the addition of Freund's adjuvant to autoantigens like brain, testes, or thyroid the effects could not have been studied systematically (*25*). Until now we have restated in immunologic terms what has been known for a long time: Certain cells or organs produce proteins with specialized functions, *i.e.*, γ-globulin, thyroglobulin, albumin, etc., and these proteins are therefore tissue specific. However, it should be possible to find other tissue-specific antigens and to define them, for almost every organ has its own function.

Our first attempts to demonstrate tissue-specific antigens were based on the assumption that the injection of whole organ or tissue homogenates or extracts into rabbits might induce antibody production against antigens specific for that organ or tissue. This assumption has proved to be erroneous, mainly because intracellular tissue proteins are poor antibody inducers. The antitissue serums so obtained usually contain antibody only against antigens that most cells have in common, and the number of antigens capable of reacting with these antiserums is always small. Recently we have turned to more homogenous cells, which could be washed to remove almost all plasma proteins.

The availability of human cells grown in tissue culture should ultimately result in the detection, isolation, and characterization of many cell-specific antigens. We have been fortunate in obtaining samples of such cells in sufficiently large amounts for immunologic studies. Antiserums were produced against H.Ep #1, H.Ep #2, H.Ep #3, H.S. #1 (*26*), Osgood's J96 and J111 (*27*), and amnion cells (1st and 2d generation). The rabbits were immunized with the saline insoluble cell fractions (*28*), and the antiserums were absorbed with the animal serums used in the tissue-culture media. Many of these antiserums reacted with antigens common to all or most cell lines with which they were tested. For example (fig. 6), an antiserum against H.Ep #3 (an epidermoid carcinoma) reacted with H.S. #1 (a sarcoma, cup B) to the same degree as with the homologous cell antigens.

The antiserum against H.S. #1 (fig. 7) contained antibody against two main components; one, which formed the line closest to the center cup, was present in all cells, whereas the other was present in H.S. #1 (cups A and D) and in H.Ep #3 (cup B), but could not be detected in the other cell extracts tested at the same concentration (20 mg./ml.). The antiserum against H.Ep #1, however, was specific in that it did not react with extracts of the other cell lines grown in tissue culture nor with the extracts of tumor specimens obtained from surgery (fig. 8). Two antiserums against first and second generation amnion cells (grown in human serum) contained antibody specific for these cells. Figure 9 shows that one of these antiserums, prior to absorption with human serum, reacts strongly with at least two serum components. The strong line at F is formed by the α_2-macroglobulin. Note that this antiserum also reacts with antigens of other cell lines grown in tissue culture, but that the homologous cells contain a specific antigen. The same antigen could also be detected by a second antiserum (fig. 10), which was absorbed with human plasma. This antiserum still reacted with 5 mg. per ml. of the homologous extract, but failed to produce a line with extracts of surgical specimens at 40 mg. per ml. The study of human cells grown in tissue culture has, however, some disadvantages. A large plant is required to produce enough cells for thorough antigenic analysis. Moreover, a certain degree of uncertainty exists as to the effects of growth *in vitro* on antigen synthesis; certain antigens may be lost, whereas others, present initially in small amounts, may now be produced in large quantities. Finally, it is conceivable that some proteins synthesized by a cell *in vitro* are antigenically different from the original. We have, therefore, studied human leukocytes and lymphocytes, which, although not as homogeneous a cellular population, may be obtained directly from the patient, often in large amounts. Many antiserums have now been prepared against human leukocytes, both normal and leukemic, as well as against leukemic lymphocytes.

It soon became apparent that potent antiserums could be produced against granulocytes. Detailed accounts of the methods used and the results obtained in this study, done in collaboration with Dr. D. G. Miller, are now being prepared for publication. Many of these antiserums contained antibody against a component (antigen #1) that was present in all mature granulocytes but not in other cell types. This is illustrated in figure 11. An antiserum against cells from a patient with chronic myelogenous leukemia is in the center cup. Cups A, B, D, and F contain extracts of granulocytes obtained from different patients with chronic myelogenous leukemia; cups C and E contain extracts from cells of patients with acute leukemia. The heavy precipitin line produced by the extracts in cups A, B, D, and F is formed by antigen 1. Note the lines produced between the cups containing the granulocyte extracts and cups C and E. Moreover, since the human plasma used for absorption of the antiserums has been dialyzed, these lines extend to the center. This phenomenon has been described elsewhere (*29*). Small amounts of the antigen, which seems to be specific for granulocytes, were also found in other cell extracts when they were used at high concentration, but this was to be expected, because these preparations contained contaminating granulocytes. However, it could not be demonstrated in human cells grown in cortisonized rats or in tissue culture. Another example of antigens that are specific for certain leukocytes is presented in figure 12. The center cup contains an antiserum against leukocytes obtained from a patient with acute leukemia. Cups A and D contain the extracts of the homologous cells, cups E and F contain extracts of cells from patients with chronic lymphocytic leukemia, cups B and C contain the Osgood J96 and J111, respectively.

A second leukocyte antigen (#3) with a relative degree of specificity can be detected with an antiserum against a cellular preparation obtained from another patient with acute leukemia. This antigen is present in considerable amounts in the homologous cells as well as in the leukocytes from several other patients with acute leukemia; however, morphologically similar cells obtained from other patients with acute leukemia, as well as cells of patients with chronic myelogenous or lymphocytic leukemia, lacked this antigen (fig. 13). In the center of the plate of figure 13 is the absorbed antiserum: cup A contains the homologous cell extract, cups B, D, and F contain extracts of cells from patients with chronic myelogenous leukemia, and cups C and E contain extracts from patients with acute leukemia. The antigen from cup A that reacts so strongly with this antiserum is, however, not leukocyte specific, since it is also present in such organs as liver, spleen, lymph nodes, and reticulum-cell sarcoma. This antiserum obviously also contains antibody against a granulocyte antigen.

Attempts to find antigens specific for the lymphocyte have been unsuccessful, though several rabbits produced good antiserums in response to the injection of cells from patients with lymphocytic leukemia. The immunologist studying complex antigenic systems is more or less at the mercy of the animals that provide him with antiserums. Different animals are known to respond differently to injections of the same mixture, and consequently, the antiserum is a reagent that is not easily reproducible. It is therefore almost impossible to communicate data on tissue antigens in a manner that will be useful to other workers. One remedy for this situation is the characterization and isolation of the tissue antigens under investigation.

A few years ago we therefore initiated a program of fractionating the soluble tissue proteins by various methods (*17*, *28*), and this work is now being continued with leukocyte antigens. Fractionation of leukocyte antigens by zone-electrophoresis on starch has shown that leukocyte antigen #1 is a protein with the electrophoretic mobility of γ_2-globulin; antigen #3 has a mobility of α_2-globulin. Moreover, the curvature of the line produced by antigen #3 indicates that it is a large molecule with a diffusion coefficient considerably less than that of 7S γ-globulin. Antigen #1, on the other hand, has a coefficient greater than that of antibody.

The leukocyte antigens can also be studied by microimmunoelectrophoresis as described by Scheidegger (*30*). In figure 14, extracts from leukocytes obtained from patients with chronic myelogenous leukemia or acute leukemia were tested with their homologous antiserums. The antigens were used at the same concentration, and the marked quantitative differences between the two extracts are apparent. The lower part of the plate contained purified serum proteins ranging from γ_2-globulin to the α-globulins; these were included to act as reference substances with known electrophoretic mobilities. The data obtained by this method are sometimes ambiguous, since some proteins, like the β-lipoprotein (cup 8) will move in agar like an α_2-globulin (*31*).

Recently we began to purify human myeloperoxidase, a green enzyme that is found in granulocytes. Using cells from patients with chronic myelogenous leukemia as the starting material, we now have preparations that are 50 to 60 percent pure, according to the spectrophotometric data of Agner (*32*). The initial green preparation (17% pure) was analyzed with the same antiserum as in figure 11, and many lines were formed by this crude sample of myeloperoxidase (fig. 15). After a second purificatory step, the enzyme was approximately 45 percent pure, and many, but not all, of the lines formed by the crude material had disappeared (fig. 16). As yet we cannot be sure whether one of the lines is formed by the myeloperoxidase. Tests with crystalline enzyme will be necessary to prove this point conclusively.

Last year (*18*) I suggested that tissue or cell-specific antigens lacking from antibody-forming cells would be the only ones capable of eliciting a detectable autoimmune response, and similar opinions have been expressed by others (*16*). Therefore, the availability of myeloperoxidase in pure form may provide us eventually with an opportunity to test the autoantigenicity of this enzyme. This would be of special interest, since myeloperoxidase is formed by cells of mesodermal origin. The hypothesis that any antigen lacking from cells involved in the formation of immune substances can be autoantigenic has the advantage of simplicity, since it dispenses with the need of explaining how, during the embryologic development, the organism succeeds in coding all the antigenic determinants that in future life it will have to recognize as belonging to itself. Moreover, the hypothesis accounts for all the available experimental data dealing with autoimmunity.

SUMMARY

The term "tissue antigen" is defined as any antigen produced by cells, organs, or tissues. The specificity of an antigen resides in the antigenic determinants of the molecule. Cross-reactions result when an antiserum contains antibody against determinants that different proteins have in common; specificity depends on the presence of antibody against a unique determinant. Examples of cross-reactions between proteins obtained from animals of different species and proteins obtained from individuals of the same species were presented, as were the features which provide these proteins with their specificity. Extracellular tissue antigens, *i.e.*, serum albumin, the γ-globulins, and thyroglobulins were discussed in some detail, as were the intracellular antigens produced by leukocytes and human cells grown in tissue culture. Preliminary data on the antigenic characteristics of crude preparations of human myeloperoxidase were presented.

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PLATES

PLATE 5

FIGURE 1.—Center cup: goat antirabbit-serum albumin; albumins of: A: rabbit; B: swine; C: human; D: rat; E: calf; F: dog.

FIGURE 2.—Center cup: rabbit antihuman γ-globulin; A and D: human γ-globulin; B: calf γ-globulin; C: rat γ-globulin; E: swine γ-globulin; F: cow γ-globulin.

FIGURE 3.—A: 19S macroglobulin; B: anti-7S globulin; C: normal human serum; D: anti-7S, 19S globulin.

FIGURE 4.—A: 19S γ-globulin; B: anti-7S globulin; C: 7S γ-globulin; D: anti-7S, 19S globulin.

FIGURE 5.—Center cup: thyroglobulin; A: Hashimoto's struma serum #V; B and D: empty; C: Hashimoto's struma serum #I.

FIGURE 6.—Center cup: anti-H.Ep #3; A and D: H.Ep #3, 20 mg. per ml.; B: H.S. #1, 20 mg. per ml.; C: H.Ep #1, 20 mg. per ml.; E: Osgood J96, 20 mg. per ml.

FIGURE 7.—Center cup: anti-H.S. #1; A and D: H.S. #1, 20 mg. per ml.; B: H.Ep #3, 20 mg. per ml.; C: H.Ep #2, 20 mg. per ml.; E: Osgood J111, 20 mg. per ml.; F: Osgood J96, 20 mg. per ml.

FIGURE 8.—Center cup: anti-H.Ep #1 serum; A: H.Ep #1, 20 mg. per ml.; B to F: surgical specimens, 40 mg. per ml.

FIGURE 9.—Center cup: antiamnion cells (not absorbed): A: amnion cells, 20 mg. per ml.; B: H.Ep #1, 20 mg. per ml.; C: H.S. #1, 20 mg. per ml.; D: Osgood J96, 20 mg. per ml.; E: H.Ep #3, 20 mg. per ml.; F: human serum (1:2).

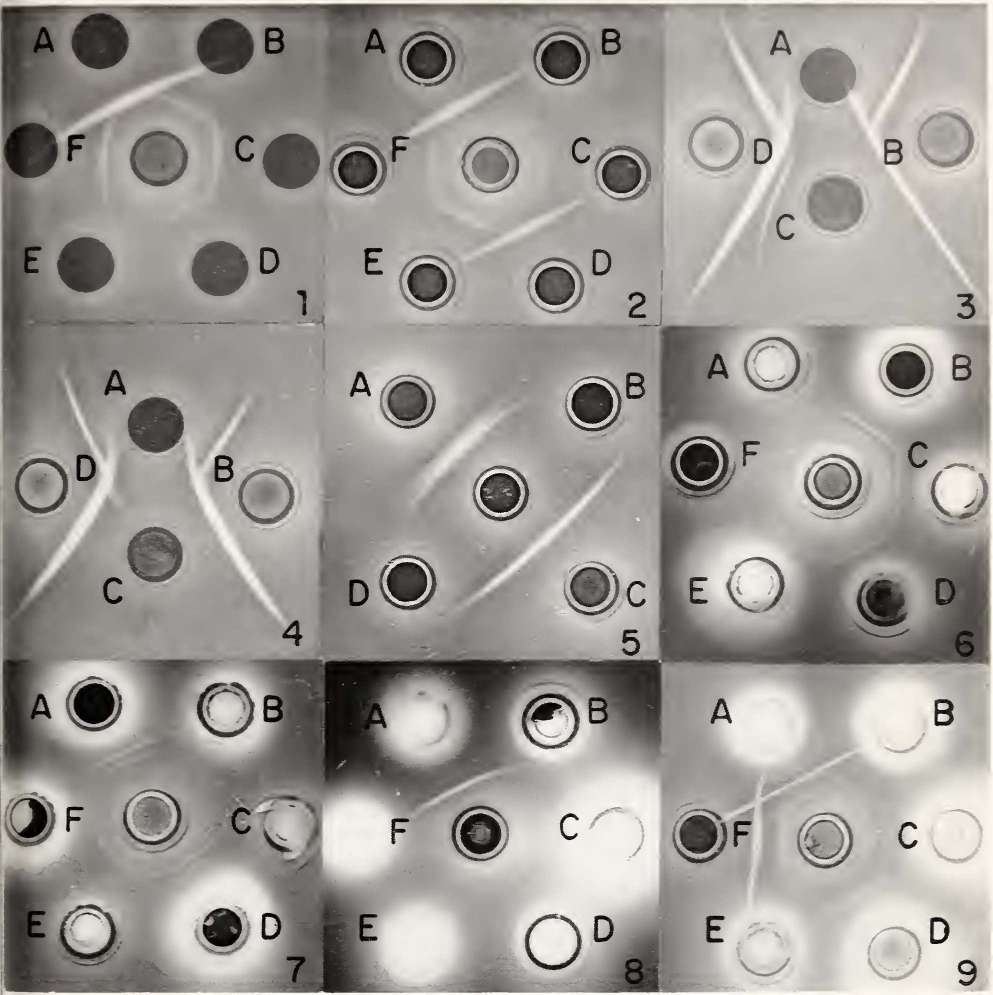
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PLATE 6

FIGURE 10.—Center cup: antiamnion cells; A: amnion cells, 5 mg. per ml.; D: amnion cells, 10 mg. per ml.; B, C, E, and F: surgical specimens, 40 mg. per ml.

FIGURE 11.—Center cup: antigranulocyte serum; A, B, D, and F: extracts of leukocytes from patients with chronic myelogenous leukemia; C and E: extracts of leukocytes from patients with acute leukemia.

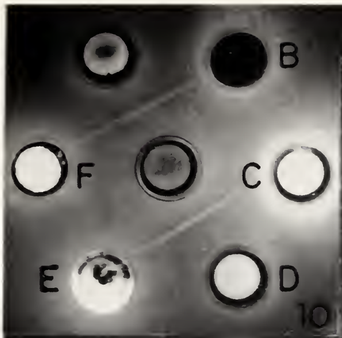
FIGURE 12.—Center cup: antileukocyte serum; A: homologous leukocyte extract, 20 mg. per ml.; B: Osgood J96, 20 mg. per ml.; C: Osgood J111, 20 mg. per ml.; D: homologous leukocyte extract, 20 mg. per ml.; E and F: chronic lymphocytic leukemia extracts, 20 mg. per ml.

FIGURE 13.—Center cup: antiacute leukemia serum; A: homologous leukocyte extract; B, D, and F: extracts of leukocytes from patients with chronic myelogenous leukemia; C and E: extracts of leukocytes from patients with acute leukemia.

FIGURE 14.—Immunoelectrophoresis of leukocyte antigens and serum proteins; 1 and 3: acute leukemic leukocyte extract; 2: chronic myelogenous leukemia leukocytes extract; 4: γ_2-globulin; 5: γ_1-globulin; 6: transferrin; 7: ceruloplasmin; 8: β-lipoprotein; A and C: antiacute leukemia; B: antigranulocyte serum; E: anti-γ-globulin; F: antitransferrin serum; G: anticeruloplasmin serum; H: anti-β-lipoprotein serum.

FIGURE 15.—The purification of myeloperoxidase. Center cup: antigranulocyte serum; A: granulocyte extract, 2.5 mg. per ml.; B: crude myeloperoxidase, 1 mg. per ml.; C: crude myeloperoxidase, 0.5 mg. per ml.; D: crude myeloperoxidase, 0.1 mg. per ml.

FIGURE 16.—The purification of myeloperoxidase. Center cup: antigranulocyte serum; A: granulocyte extract, 2.5 mg. per ml.; B: myeloperoxidase, 45 percent pure; C: myeloperoxidase, 45 percent pure (1:2); D: myeloperoxidase, 45 percent pure (1:4).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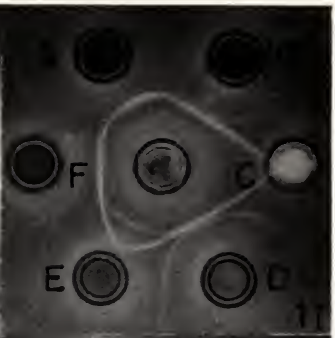
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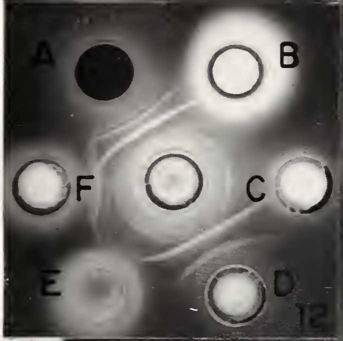
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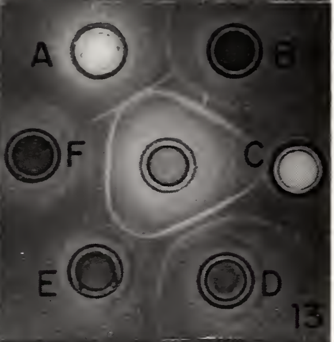
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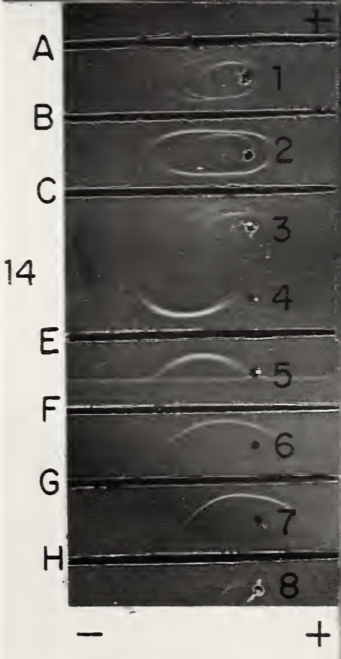
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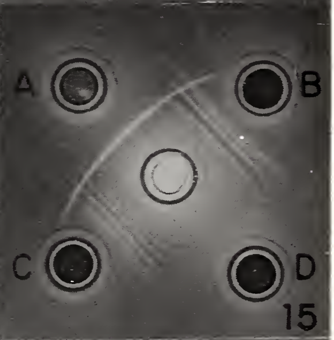
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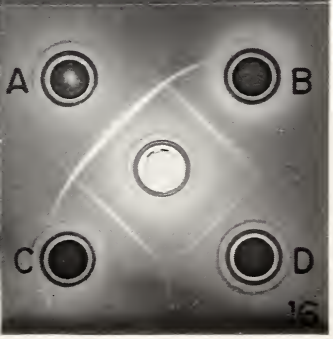
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KORNGOLD

Ontogenesis of the Immune Response[1]

James D. Ebert *and* Louis E. DeLanney,[2, 3] *Department of Embryology, Carnegie Institution of Washington, Baltimore, Maryland, and Department of Biology, Wabash College, Crawfordsville, Indiana*

At a recent conference on "Fashions in Learning," Amberson (*1*) argued that, in the world of science, fashion is a prevailing mode of thought or action determined by a recent innovation. A new technique or a seminal idea sweeps us toward renewed discovery, resulting in a wave of attention and emphasis, which can be construed as fashion without implying derogation. Yet in the wake of fashionable movements, difficult, even critical, questions are neglected, which often escape attention through several succeeding waves. So it happens that despite our preoccupation with immunobiologic concepts, generated by the intense interest in actively acquired tolerance (*2–4*) and the graft-*versus*-host reaction (*5–9*), we have failed to reach even the first level of understanding the mechanisms whereby the capacity for an immune response is established during embryonic development. In fact a pattern has emerged in which embryologic questions frequently are dismissed with an introductory statement, such as it is generally accepted that the embryo is incapable of producing antibodies, or it is generally agreed that the internal environment of newborn animals is not favorable for antibody production (*10*, *11*). We are reminded always to cherish our exceptions; it is, in fact, the intent of the authors to examine the exceptions to the rule, that the embryonic environment is not favorable for immune reactions, in an effort to emphasize how much is to be gained by a concerted attack on the question of maturation of the immune response. With Hamburger (*12*), we argue that the early formative phases are the decisive ones, for at birth (or shortly before or after birth, varying with the species) there exists a population of mesenchymal cells capable of responding to an antigenic stimulus. Clearly we may speak of these cells as having differentiated, for they have a capacity which distinguishes them from their fellows; yet the *further differentiation* of a given cell in that population depends on the specific antigenic stimulus to which it reacts. In considering these questions we shall retain the distinction adopted by Ebert (*13*) between immune responses mediated by circulating antibodies and those mediated by cells (*cf. 14–17*). The distinction may be regarded as arbitrary, but whether it will prove ultimately to be unnecessary is problematic [*cf.* (*18*) and (*19*)]. It will remain a useful concept, at least until it has been shown that specific homograft immunity can be transferred passively by immune serums. We shall not attempt to be exhaustive in coverage; reference will be made to the works of Billingham (*4*), Ebert (*8*, *13*, *20*), and Schechtman (*21–23*), as well as to two collections of papers, "Symposium on Antibodies: Their Production and Mechanism of Action" (*24*) and the "Third Tissue Homotransplantation Conference" (*25*).

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[1] Presented at a symposium on normal and abnormal differentiation and development, held in celebration of the 30th anniversary of the Roscoe B. Jackson Memorial Laboratory, June 15–17, 1959, at Bar Harbor, Maine.

[2] Dr. DeLanney's research was supported in part by grant G-1381 from the National Science Foundation and grant RG-5619 from the Public Health Service.

[3] We acknowledge the technical assistance of William Duncan, Edward Johnson, and W. D. McCleary; and thank Drs. A. M. Mun and P. J. Van Alten who permitted us to read manuscripts prior to publication. Discussions with J. R. Coleman and Dr. R. F. Ruth have been helpful.

THE ONTOGENY OF TRANSPLANTATION IMMUNITY

Homografts, heterografts, and, not infrequently, xenoplastic grafts are accepted by embryos and maintained throughout most, if not all, of the embryonic period. Interspecific and intergeneric combinations are common in studies with chimeras, in which donor and host tissues may be distinguished by differences in cell size, chromosome number, or pigmentary features that permit assessment of the relative contributions of the graft and host (*26–28*), *e.g.*, in studying the specificity of embryonic induction or in analyzing the fate of mixtures of chick and mouse cells *in vitro* (*29–31*). Ebert (*13*) has stressed that in the older studies most of the experiments were terminated during embryonic life; hence incompatibilities were the exception rather than the rule. When chimeras were permitted occasionally to develop beyond embryonic life, relatively little attention was given to differences in the results with homografts as compared to heterografts. In the latter, the destruction of the graft by the host was commonly encountered in birds and mammals and frequently in teleosts and amphibians. One of the first detailed analyses of the development of incompatibility between amphibian hosts and grafts as related to host age is that of Eakin and Harris (*32; see also 33*), who placed the onset of host antagonism at about the time of metamorphosis. Now, Hildemann and Haas (*34*) have presented a preliminary account of a study of homotransplantation immunity in the developing bullfrog, *Rana catesbeiana*. Skin homografts were made reciprocally between pairs of bullfrog larvae at the same standard stages under controlled temperatures. Autografts served as controls. Tailbud homografts were exchanged successfully between larvae at stage 24 or earlier (less than 2 weeks of age). Subsequent skin homografts from the same 16-week-old donors showed greatly prolonged survival, which indicates the induction of immunologic tolerance by the first-set grafts. Older larvae (stages 25–29) rejected homografts at 25°C., with median survival times of 11 to 14 days and 5 days for first- and second-set transplants, respectively. When the animals were kept at 15°C., first-set homografts survived for about twice as long, but the median survival time of the second-set transplants was again reduced. Hildemann and Haas conclude that mechanisms of transplantation immunity are mature beyond 8 weeks of age.

Grafts of avian and mammalian tissues succeed in the chick embryo (*35–40*). When limbs are grafted from bird embryos of one species to those of another, they are destroyed almost without exception by the 6th week after hatching (*41*). Conversely, from observations by Willier and Rawles (*42*), Weiss and Andres (*43*), and others, we know that neural-crest cells transplanted into embryos of the same species may survive beyond hatching and even into adult life. Skin transplants between newly hatched chicks of different breeds frequently succeed, but the incidence of successful grafts decreases with age (*44*, *45*). The absence of incompatibility reaction results primarily from a failure on the part of the host to react and not on a lack of antigenic stimulus, since grafts of adult tissue persist in the embryo almost equally as well as grafts from embryonic donors. Although the ontogeny of isoantigens (individual-specific antigens) has not been studied, it is known that many embryonic tissues are antigenic (*13*, *20*, *46*, *47*).

Billingham (*4*) and Ebert (*8*) have emphasized that maturity in transplantation immunity need not correspond temporally with birth. At one extreme of the time scale, the fetal sheep can reject skin homografts as early as 40 days before birth (*48*); at the other extreme, immune reactions develop slowly in the rat—as much as 2 weeks after birth during which not only homografting, but also parabiosis can be effected (*49*, *50*). The duck also attains immunologic maturity relatively late (*51*, *52*) in contrast to the chick and mouse, in which immune mechanisms are fixed shortly after birth. Billingham (*4*, p. 577) has presented a table showing the upper limit of the tolerance responsive period of living homologous tissue cells in nine species. There is one obvious weakness in the foregoing discussion, namely, that the critical periods for the several species which were studied are derived from experiments using different antigenic stimuli. At least in theory we are confronted with the distinct possibility that the embryo or newborn animal may acquire the ability to recognize different antigens at different times. Billingham (*4*) has remarked that newborn calves react against skin homografts with a vigor like that of adult cattle, yet they may still become tolerant of *Trichomonas foetus* antigen (*53*). Billingham also has discussed the striking difference of about 12 days in the critical period in tolerance to homologous tissue cells and bovine serum albumin.

PRODUCTION OF HUMORAL ANTIBODIES

We have long been told that most embryos and newborn animals do not form antibodies in response to the injection of antigens; the capacity to respond matures slowly. Negative findings must be stated with the reservation that our ability to delineate the synthesis of any specific protein, including antibody, is limited by the sensitivity of our techniques. It can be said only that the ability to produce humoral antibody in response to an antigenic stimulus develops first about the time of birth (*54–57*). Allowing for species variation, and for the fact that only a few of the more common animals have been studied, and with few antigens, we may state that the antibody-forming system reaches a state of functional development within a few days after birth, at which time antibody can be formed in small quantities. The system matures rapidly; in the chick "serologic maturity" is reached by about 6 weeks post hatching. Although the wisdom of attempting to correlate the production of circulating antibody with the onset of transplantation immunity may be questioned, the striking parallelism in the time course of their development must be noted.

But what of the reports that embryos contain "natural antibodies," *e.g.*, the report of Green and Lorincz (*58*) who attribute the degeneration, beginning at about the 17th day, of Krebs mouse ascites-tumor cells in the chick embryo to the formation of a serum protein with the properties of a natural antibody. Perhaps we use the expression natural antibody to mask our complete lack of understanding of the serum proteins (if indeed proteins are the only molecular species concerned) that are toxic to heterologous cells (*46*). The problem is no less intriguing, but we may do ourselves a disservice by linking it too closely with the production of antibody. Also, we must not disregard the fact that in birds the transmission of antibodies from the hen to the offspring through the yolk has been established firmly. After the demonstration by Klemperer (*59*) of the transmission of tetanus antibody, it was shown that antibodies against *Spirillum gallinarum* (*60*), Rous sarcoma virus (*61*), Newcastle disease virus (*62*), and *Salmonella pullorum* (*63*) were transmitted by the same route. Warren and Borsos (*64*) have shown by immunologic methods that chick serums contain at least two factors involved in the lysis of the *Crithidia* of *Trypanosoma cruzi:* a heat-stable factor, probably an agglutinating and a sensitizing antibody, and a heat-labile factor, probably complement. The antibody in the serums of mature fowls is transmitted to the yolk and thence to the blood of the embryo. *It is greatly reduced or lost after hatching and regained upon immunologic maturation.* Eggs, taken from hens reared under usual laboratory conditions and containing the serum antibody, which were incubated, hatched, and grown in a germ-free environment, had the antibody in their serums until shortly after hatching. Under standard laboratory conditions the antibody was lost and *did not reappear.* Borsos and Kent (*65*) have advanced preliminary findings correlating this pattern with progressive changes in the γ-globulin content in serums of developing chicks. Confirming earlier findings (*66*), low levels of γ-globulin were detected in the serum of the 11-day embryo, with a progressive increase recorded throughout the embryonic period. After absorption of the yolk at hatching, the γ-globulin levels decline until the birds reach immunologic maturity when the levels rise. In contrast, chicks reared under germ-free conditions reveal low levels of γ-globulin, which parallels the results with agglutinins for *T. cruzi.* The observations imply a close correlation between serum γ-globulin levels and the antibody; chickens reared under germ-free conditions do not *replace* maternal antibody with their own γ-globulin earmarked as specific antibody for *T. cruzi.* Is it meaningful that a low level of γ-globulin does reappear? It is virtually impossible to assess the significance of this preliminary finding, but it suggests that the synthesis of most γ-globulin does follow antigenic stimuli. The passive immunity of the young animal is treated fully by Brambell (*67*). For a comprehensive account of serum proteins and related subjects in germ-free vertebrates, *see* (*68*).

Development of Antigenic Receptors *Versus* Immunologic Responses

Until now we have treated the development of the immune response as if the capacity to receive an antigenic stimulus and the capacity to respond were inseparable in space and time. We must now raise the question whether these two stages are, in fact, separable. We propose to treat the question more fully in subsequent sections, and emphasize only its significance at this time. The argument that the production of antibody requires teamwork of two or more cells of different types has been advanced repeatedly (reviewed, *69*, *70*). Evidence to be presented suggests that, although the chick embryo is incapable of a significant first-set homograft reaction before hatching, during this period it is capable of being sensitized by the antigenic stimulus of a first-set graft and can respond vigorously to a second stimulus as early as the 14th day.

EXPERIMENTAL ALTERATION OF DEVELOPING IMMUNE MECHANISMS

Actively Acquired Tolerance

Although the immune mechanisms of the embryo are incomplete, during their maturation they are susceptible to experimental alteration. As Billingham (*4*) has stated, "They may undergo a highly specific adaptive change of considerable, if not permanent, duration, . . . which . . . expresses itself in later life as a specific inhibition or impairment of their capacity to respond to a later challenge with the (specific) antigen. . . ." Although the theory of acquired tolerance was proposed by Burnet and Fenner (*71*), it has been emphasized justly (*4*, *72*) that this theory of far-reaching significance had its factual cornerstone in the observation by Owen and coworkers that twin cattle showed identical blood types more often than would have been expected from estimates of the occurrence of identical twins. One pair of twins had different sires yet identical blood types, which consisted of a mixture of two distinct cell populations. A study of higher multiple births, including a set of quintuplets (*73*), was consistent. The placental anastomosis of blood vessels, which is known in cattle twins (*74*), led Owen to advance the hypothesis that the identical blood types resulted from an exchange of reciprocally "transplanted" hematoblasts. He later argued that ". . . the cells were established and retained as intact units, developing autonomously as a function of their own genotypes; there was never any indication that exchanges of determinants at the subcellular level had produced 'transformation' for some of the gene-controlled antigens in which the members of a twin pair differed, and not of others" (*72*). Spontaneous transfer of hematoblasts occur in sheep (*75*) and man (*76*); the result has been achieved experimentally in rats (*72*) and embryonic chicks (*51*).

Burnet and Fenner predicted that the exposure of an embryo to foreign cells should result in the inability of the animal to respond immunologically against those cells in later life, after its faculty for immunologic response had developed. This postulate was tested by Billingham, Medawar, and their coworkers, who found that the majority of nonidentical twin calves would accept homografts of each other's skin, whereas grafts exchanged between siblings were rapidly destroyed. The reaction was highly specific, for a calf accepted homografts only from its twin and from no other donor (*77*, *78*). Shortly thereafter the phenomenon which occurs in twin cattle was reproduced experimentally (*2*, *3*) in mice. We do not propose to retrace the course of these experiments, for the findings and their implications have been re-examined recently by Billingham (*4*) and Ebert (*13*). "Actively acquired tolerance" is defined as a specific reduction or suppression of immunologic reactivity as the result of exposing the animals to antigenic stimuli before the maturation of the faculty of immunologic response. Tolerance of homografts is achieved by exposing embryos or newborn animals to a dose of foreign homologous cells, which in adults would cause them to become immune. What is the optimal time for the induction of tolerance? Billingham (*4*) has concluded that ". . . no rigid correlation or causal relationship exists between hatching or birth and the end of the tolerance responsive period. The important fact appears to be the state of maturity of the animal at term."

Tolerance is the expression of a modification of the host's immune system; it does not appear to be the result of a change in the transplant (*4*, *13; cf. 79*). Billingham (*4*) and Ebert (*13*) consider the evidence on the specificity of tolerance, but with a difference in emphasis that may be meaningful. That experimentally induced tolerance is individually specific or isoantigenically specific seems well established; if tolerance is complete, an animal will accept only grafts having the same genetic constitution as the cells which evoked the tolerant state. On the other hand, as Billingham makes clear, animals made tolerant early in life are incapable of discriminating immunologically *between the different tissues* of the original donor. Tolerance evoked by treatment with spleen cells is effective with later grafts of such tissues as skin, adrenal cortex, ovary, and thyroid (references in *4*, *25*). In his searching analysis Billingham concludes that ". . . all the living tissue cells of the body of an individual have exactly the same complement of transplantation antigens." Ebert (*13*), however, in reviewing the same findings, but of course without the same personal familiarity with the problem, asked whether the question of the lack of tissue specificity should be considered resolved. In viewing the question from the standpoint of the nature of the inoculum, we must ask *whether leukocytes must be included in the inoculum for homograft tolerance.* For example, in Billingham's table 2 (*4*, p. 582), with only one possible exception the tissue-inducing tolerance contains cells with immunologic or potential competence. If immunologically competent cells must be included, what is their role? It may be of more than passing interest that in studying the fate of the inoculum, Billingham and Brent (*4*, *5*) succeeded in demonstrating the persistence of spleen cells in the spleens, lymph nodes, thymuses, and bone marrow of tolerant mice. Since tolerance represents a specific failure of the host's immunologic mechanisms, the nature of the reaction must be sought in the host's immunologically competent tissues. Do we find clues in the phenomenon of predominant localization of cells and subcellular particulates (*7*, *8*, *13*, *50*, *80–82*)? From this point of view the precise relation between homograft tolerance and the graft-*versus*-host reaction, to be discussed later, is unclear. The graft-*versus*-host reaction appears *not to be causally related* to acquired tolerance, the involution of the host's lymphoid tissue in the graft-*versus*-host reaction being generalized rather than specific. As we have seen, acquired tolerance is an individual-specific phenomenon. Moreover, animals may be made tolerant by injecting normal embryonic spleen cells which are incapable of destructive action on the host (*83–85*). The nature of the inoculum is one of the most critical experimental targets.

Evidence that tolerance may be induced by living cells of heterologous origin has been summarized effectively by Billingham (*4*, *see also 86*); significantly each of the examples cited involves the transfer of heterologous blood or spleen cells. The question remains, however, whether actively acquired tolerance, as originally defined, is restricted to transplantation immunity or whether it is a phenomenon of more widespread occurrence and significance. Transplantation immunity appears to differ from the immunity to soluble antigens, as it is cell-borne and cell-mediated, with no cause and effect dependence on circulating antibody having been established. Until recently most of the studies on acquired tolerance to homografts were performed in chicks and mice, whereas studies on the depression of serum antibody response were carried out in rabbits, which make direct comparisons difficult. The difficulty has been overcome, in part, by recent studies on immunologic unresponsiveness in the chick (*87–89*), but the relation of acquired tolerance to soluble antigens and immunologic paralysis (*90*) remains obscure (reviewed in *13*, *72*). Whether immunologic tolerance of homografts is "nothing more" than paralysis as a result of continued proliferation of donor cells in the host remains to be determined. Worthy of attention, however, are the first studies of the induction of immunologic unresponsivness to tissue-specific antigens.

In the study of tissue specificity, a limiting factor is the presence in each tissue of common antigens. Often these constituents which may be shared with several tissues are strongly antigenic. After an antiserum is produced against antigens of one tissue, the antiserum must be absorbed repeatedly with blood and other tissue extracts to remove antibodies against the common antigens. This tedious method is unsatisfactory because of the high concentration of nonspecific, relative to specific, antibody present. Feldman and Yaffe (*91*) have advanced the possibility that the production of specific antibodies to antigens of one organ might be improved in animals which had been made tolerant of common antigens. If the objective is an antiheart serum, the animals destined to produce the antibody might be injected in the fetal or neonatal period with brain or liver, or with a mixture of antigens. They found that the introduction into neonatal rabbits of brain or heart antigens inhibits the later development of the capacity to produce cross-reacting antibodies. Specific antibody response to selected types of antigenic components within the complex of tissue antigens thus seems entirely possible.

The induction of immunologic tolerance raises several questions regarding the nature of the phenomenon, yet to attack them in systems using living cells is difficult. Among these questions are the following: How small a dose of antigen is required? Is the continued presence of the antigen necessary? When animals are exposed to a mixture of antigens at birth, do they become equally tolerant to all antigens? These questions can be approached by the use of tissue antigens; for the last question, the serum-agar diffusion techniques, *e.g.*, those of Ouchterlony, may prove to be especially suitable. Wyttenbach (*92*) injected newborn rabbits with saline homogenates of chicken liver or spleen. At 11 weeks, the animals were challenged with the homologous homogenate. By precipitin tests, positive control animals (previously uninjected) gave titers of 2000 and 6400, respectively; the corresponding experimental groups gave average titers of about 200. In a different group of rabbits not challenged until later (20 weeks), response was suppressed but not as greatly. When the spleen-antispleen systems (control and experimental) were studied by the Ouchterlony technique, serums of all experimental ("tolerant") rabbits gave several *more* bands than the positive controls, Wyttenbach thought that the previously uninjected animals responded strongly to a few major components, whereas, in the treated group, the response toward these components was eliminated or reduced to the extent that the rabbits then responded to a variety of lesser components.

Immunologic Tolerance and Auto-Immunization

It is a fundamental premise that for a substance to be antigenic it must be foreign to the test animal. Burnet and Fenner postulated that each cell acquires during its development a "self-marker" component, which prevents the organism from making an immune response to constituents of its own cells. But what of the "exceptions to the rule"? If autologous or homologous tissues are modified slightly they may stimulate an immune response. Moreover, although the alteration has been sufficiently great to make the inoculum antigenic, the antigens are still closely related to the proteins of the host, so closely related that the immune reaction which is elicited may not distinguish the altered from the normal form. As a consequence the normal host tissue may be destroyed. Or, if tissue-specific molecules are not produced until after the animal's immune mechanisms have developed (thereby eliminating an opportunity for exposure during early development) or if they are formed in what Billingham has called "physiological quarantine," they should be auto-antigenic. Billingham (*4*) cites several examples: in the former category, spermatozoa, and in the latter, brain antigens, lens proteins, and thyroglobulin. Only two of these examples will be considered here, and in inverse order. It has been shown that the injection of normal nervous tissue combined with adjuvant into animals of several different species results in allergic encephalomyelitis. A number of other serious diseases may be caused by auto-immunization, *e.g.*, acquired hemolytic anemia, rheumatic fever, rheumatoid arthritis, glomerulonephritis, and sympathetic ophthalmia. Patterson (*93*) injected newborn rats with a homogenate of heterologous spinal cord; the result: a marked decrease in their capacity to develop allergic encephalomyelitis after injection of the antigen 8 to 10 weeks later. In keeping with the foregoing discussion, the protective effect of the inoculum may be ascribed to its ability to confer tolerance with respect to the specific antigens in nervous tissue which are responsible for the response.

Cytologic and functional degeneration of the guinea-pig testis results from the injection of homologous testicular tissue combined with adjuvant (*94–98*). Studies of the rabbit and rat have indicated that induced testicular destruction is not limited to the guinea pig. Moreover, it has been shown in a parallel investigation of the female guinea pig that, while the ovary is not affected by the injection of homologous ovary and testis, the capacity to bear young is reduced, possibly as a result of an anaphylactoid response involving the uterus of the injected female animals. In both sexes and in all the species studied, the response to the administration of gonadal-tissue homogenate and adjuvant indicates that an immune reaction is involved.

The antigenic component begins to appear in quantity in the testes of guinea pigs between the 1st and 2d months after birth (the time when secondary spermatocytes are being transformed into mature sperm). Antigenicity appears to be associated with intensely staining polysaccharide granules in or near the acrosome. The sperm of the guinea pig and of man possess acid-fast characteristics similar to some mycobacteria. The sperm of other forms, *e.g.*, opossum, rabbit, rat, and rooster show little, if any, acid-fast stainability, an observation which may prove important in explaining the relative ease with which aspermatogenesis can be induced in some forms and not in others. Adjuvant without bacteria (incomplete adjuvant) is incapable of mediating testicular destruction. It is believed, therefore, that the bacterial component participates in the reaction. Acid-fast organisms, *Mycobacterium tuberculosis* or *Mycobacterium butyricum*, are employed in adjuvant to induce aspermatogenesis, which suggests that acid-fastness is related to the ability of bacteria to act as mediators of aspermatogenesis. If the acid-fast material is an essential attribute of the bacterial component, then its removal should render bacteria ineffective. Katsh (*95–98*) injected male guinea pigs with a variety of acid-fast and nonacid-fast bacteria in adjuvant. Other groups of guinea pigs were injected with antigen plus adjuvant containing a variety of chemical components extracted from acid-fast and nonacid-fast bacteria. Acid-fast bacteria were more potent than nonacid-fast bacteria in mediating aspermatogenesis. Tubercle bacilli that have been rendered nonacid-fast are incapable of mediating testicular damage. The specific chemical factor from bacteria that can replace bacteria in adjuvant appears to be a lipopolysaccharide; lipopolysaccharide from *M. tuberculosis* is more potent than that obtained from nonacid-fast bacteria.

Although sperm-agglutinating and sperm-immobilizing antibodies appear in the serums of animals injected with sperm or testicular extract plus adjuvant, there are sound reasons (reviewed in *7*) for believing that these circulating antibodies are not instrumental in inducing aspermatogenesis. To cite only one argument, aspermatogenic activity cannot be transferred passively by the transfusion of serum of treated animals. We may inquire, however, whether aspermatogenesis is related to the reaction by which a homograft is destroyed. Might transference of immunity be achieved by a massive transfer of cells from lymph nodes draining the site of injection? The lymph nodes of adult animals injected with testicular homogenate have been removed and implanted into secondary male hosts. Although it is stated that the nodes have persisted, there has been little testicular change. Katsh has tried also to induce aspermatogenesis by transferring suspensions of lymphoid cells from sensitized guinea pigs to uninjected males, but without consistent success. It must be made clear, however, that the experiments were not performed in inbred animals; consequently it is highly probable that the negative findings are not meaningful. Evidence of the nature of the phenomenon may be sought in another way. If an immune reaction is involved, then it should increase with the age of the recipient animal. Patterson's findings raise the question whether the injection of testicular homogenate plus adjuvant into embryonic guinea pigs might induce tolerance, so that the animals would fail to become aspermatogenic when challenged after birth. The guinea pig is among those species which mature early, at least in many respects, and it might be expected that tolerance would not be bestowed by an injection after birth.

Serum Antibody and γ-Globulins in Young Animals After Transfer of Ribonucleoprotein From Adults

Sterzl and Hrubesova (*99*) made use of immunologically unresponsive newborn rabbits in experiments, which, although they are yet to be confirmed fully, suggest that it may be possible to transfer "antibody-forming capacity" into unimmunized recipients by injecting nucleoproteins which were prepared from bone marrow and spleen and taken from immunologically competent animals shortly after the administration of an antigen. The basic experiment first should be reviewed: Heat-inactivated *Salmonella paratyphi* was injected into a series of adult rabbits; after 24 and 48 hours the spleen was removed. From the spleens of one group, nuclear and cytoplasmic fractions were isolated; from a second group DNA- and RNA-proteins were extracted. These fractions were injected into unimmunized animals, both adults and 5-day-old rabbits; controls included 5-day-old rabbits injected with antigen alone. The latter produced no detectable antibody during a period of 27 days; in contrast, 5-day-old rabbits injected with nucleoprotein from immunized donors produced antibody within 2 days. In discussing the significance of the findings, Ebert (*8*) stressed the similarity to the report by Kramer and Straub (*100*) that noninduced cells of *Bacillus cereus*, when pretreated with RNase and 1 M NaCl, form penicillinase upon the addition of RNA from previously induced cells, amino acids, purines, and pyrimidines, in the absence of the inducer. RNase destroys the efficacy of the RNA in mediating the transfer. The question arises, therefore, have Sterzl and Hrubesova transferred an "activated" nucleic acid or nucleoprotein? Or is a simpler explanation possible? Has an antibody been transferred? The latter has been treated effectively by Hrubesova, Askonas, and Humphrey (*101*). Inasmuch as all "normal" rabbits contain weak antibodies to *Salmonella*, it was considered possible that nucleoproteins taken from rabbits only 24 hours after immunization might contain antibody. Experiments were performed, therefore, to use techniques designed to release antibody and to detect it even in small quantities. After the addition of RNase and carbonate buffer, *p*H 9.5, no agglutinins were detected by several techniques. If the earlier findings are to be explained as passive immunity, then the antibody must be present in trace amounts and in a masked form from which it is released in the 5-day-old rabbit. Although subject to the usual reservations concerning negative findings, the data do suggest that another explanation must be sought. Additional experiments by Hrubesova *et al.* permit the tentative conclusion that the injected ribonucleoprotein stimulates γ-globulin synthesis in the young rabbit. The incorporation of C^{14}-labeled amino acids into plasma proteins was studied in untreated and treated 9-day-old rabbits. All the serum proteins studied (albumin, α-, β-, and γ-globulin) were labeled, and except for γ-globulin were similar in both groups. Incorporation in the γ-globulin fraction of experimentals was increased significantly over that of the controls. Moreover, the spleens of the experimental animals were 10 to 40 percent heavier than those of the controls. Plasma cells were too few to be detected. Despite the clear evidence of increased labeling in the γ-globulins, the techniques proved to be too insensitive to permit the investigators to determine whether the specific *antibody* was formed from the amino acid pool under the influence of an activated RNA.

Van Doorenmaalen (*in 102*) has begun a study designed to determine whether the techniques of Sterzl and Hrubesova may be applied to (1) other antigens and (2) other species. For his initial experiments he selected crystalline, bovine serum albumin (BSA) as the antigen and the chicken as the recipient, the ability of the chicken to produce precipitins to BSA being exceptional (*56*, *57*). The preliminary findings have been negative; they are described briefly in view of the burgeoning interest in the phenomenon. Three principal types of experiments have been conducted. All have one feature in common, *viz.*, the DNA-proteins (DNA-P) and RNA-proteins (RNA-P) employed have been extracted, according to the procedure described by Sterzl and Hrubesova, from the spleens or bursae of Fabricius of adult chickens immunized 2 days previously. (1) Nine-day chick embryos of the White Leghorn strain were injected intravenously with BSA (1–4 mg. per egg) to evoke tolerance for that antigen. Twenty-two tolerant chicks (16–31 days post hatching) were injected with 4 separate preparations of spleen DNA-P and RNA-P (0.3–14.0 mg. nucleic acid and 3.5–80 mg. protein/kg. of body weight) combined with BSA (30–60 mg./kg. of body weight). Antibodies could not be detected at 24 or 48 hours after the injections. (2) In the event that the condition of tolerance might interfere with antibody formation under these conditions, 16 juvenile chickens (about 6 weeks of age) were injected with 7 separate preparations of spleen DNA-P and RNA-P in dosages comparable to those cited. Again no antibodies were detected 24 to 48 hours after the injection. (3) In young chicks the bursa of Fabricius appears to be important in antibody formation. Thus it was chosen as an additional source of nucleoproteins. Ten, 1- to 4-month-old, juvenile chickens were injected with RNA-P. Of these 6 received bursa RNA-P; 4 others were inoculated with bursa plus spleen RNA-P. Antibodies were not detected. In view of the many variables in experiments of this type, the preliminary nature of the work must be reiterated; nevertheless antibodies have been detected in *none* of 48 animals studied, with the use of a highly effective antigen-precipitin system. It must be emphasized, also, that by precipitin techniques, Van Doorenmaalen could not demonstrate the occurrence of antibodies against the injected nucleoproteins; the question arises, therefore, whether the nucleoproteins may be destroyed by serum nucleases. Jankovic, Isakovic, and Horvat (*103*) consider the latter possibility in presenting findings in agreement with those of Van Doorenmaalen. Jankovic *et al.* injected human γ-globulin into adult chickens; by the Coomb's technique, antibodies with titers of 1/1000 to 1/3000 were detected. After 3 days, the spleens of the adult chickens were removed and a ribonucleoprotein fraction was extracted. It contained neither detectable antibody nor antigen. This ribonucleoprotein was injected into two groups of young chicks, 5 and 12 days post hatching, respectively. When these chicks were bled at 1, 3, 6, and 10 days after the injection, no antibodies to human γ-globulin could be detected. Moreover, no antibodies were induced against the ribonucleoprotein. Jankovic *et al.* state that they were able to demonstrate RNase in the serum of adults and young chicks. Stavitsky (*104*) also has been unable to transfer antibody formation by nucleoprotein fractions.

The Graft-*Versus*-Host Reaction

The graft-*versus*-host reaction is produced by transplanting cells, capable of an immune response, to recipients which are immunologically unresponsive, *e.g.*, either embryos or irradiated adults (in which the hematopoietic centers are destroyed). The transplanted cells are not rejected, but instead are stimulated by antigens of the host against which they now act in a characteristic homograft reaction. As in a typical homograft reaction, the severity of the effect is dependent, at least in large part, on the genetic relationship between the donor and recipient. The graft-*versus*-host reaction, which was predicted from theoretical considerations prompted by incidental observations in studies of the destruction of kidney homografts (*105–107*), has been verified experimentally (*5–9*, *108*). We propose to consider the graft-*versus*-host reaction only in relation to our principal question, *viz.*, the ability of the embryonic environment to support immune responses, and leave the treatment of the phenomenon as it occurs in irradiated animals to others (*109–112*). Moreover, we shall center our attention on the chick embryo, and discuss the reaction in the larval salamander (*6*) and mouse (*5*) only for emphasis.

In analyzing the effects of chorio-allantoic grafts of adult chicken tissues on the differentiation and growth of the homologous organs of the recipient, DeLanney and Ebert (*in 7*, *8*) observed that after grafts of adult spleen, the spleen of the host frequently undergoes a striking degenerative change characterized by the destruction or modification of the vascular bed, which leads to a stasis in the flow of granulocytes, their accumulation in cystic masses in the spleen, and eventual death of the embryo or hatched chick. In fact, the cystic condition of the spleen occurs with a frequency approximating that of the death of chicks at or shortly after hatching. The time course of the reaction and the conditions resulting in its expression led DeLanney and Ebert to postulate that the transplanted adult spleen produces an immune response directed against its host, with the primary locus of action the vascular bed of the host.

Simonsen (*9*) demonstrated deleterious effects in the host spleen after the intravenous injection of leukocytes or spleen cells of juvenile or adult chickens into the 18-day chick embryo, and concluded that splenic enlargement and subsequent damage are due to the colonization by donor cells. Moreover, he has been able to propagate the cells through nine consecutive passages without significant loss of activity. The recipients have to be of an age in which tolerance of homologous cells can be induced; the donors must be old enough for an immune response. Further evidence is presented, *e.g.*, experiments in which 11-day or 18-day embryos of White Leghorn stock were inoculated intravenously with spleen cells of 18-day Brown Leghorn embryos. (The group injected on the 11th embryonic day comprised only 5 animals.) When sacrificed at 3 days after hatching they showed no anatomic changes. The group injected on the 18th embryonic day contained 31 animals, 14 of which were likewise sacrificed at 3 or 4 days after hatching and were found to be normal. Transplantation of embryonic chick spleen cells was ineffective. Simonsen also found that leukocytes of other avian species, pigeon and turkey, did not cause splenic damage in the chick. Although this observation is difficult to reconcile with the graft-*versus*-host hypothesis, there is ample precedent for it, in addition to partial verification from later studies. For example, Ebert (*7*, *8*, *80*, *113–115*) pointed out that grafts of mouse spleen had no effect on the spleens of chick-embryo recipients; transplanted duck spleen was less effective than homologous spleen. Mun, Kosin, and Sato (*116*) implanted fragments of turkey, pheasant, rat, and guinea-pig spleen on the chick chorio-allantois. The mammalian spleens were completely ineffective in producing an impact on the hosts' spleens; the turkey and pheasant spleens were only slightly effective.

These observations provide a further demonstration of graft-host interaction, and clearly suggest the passage of host substances capable of antigenic stimulus. Thus, there is further support to the idea of a continuous circulation of specific products in the embryo. All these findings were the results of studies that culminate during the latter half or last trimester of the developmental period. The question arises whether similar interactions are possible in the first half of the incubation period. Are the host tissues capable of a sufficient antigenic stimulus? Ebert (*7*, *8*), who made grafts of adult chicken spleen to the coelom of the 4-day chick embryo, has answered this affirmatively. Grafts were made to 4-day embryos which were permitted to develop until the 10th day, when the host spleens were harvested and used as the donor tissue for a second set of grafts. The experiment was terminated after the fourth set of grafts was recovered. On the average the host spleens were more than 5 times larger than were appropriate controls. Histologic analysis of the host spleens argues against the transfer of large populations of adult cells. However, the transfer of a seed population of graft cells is indicated clearly. Since the growth-promoting activity is not diluted by serial passage, but is maintained relatively constant, the observations may be explained in part by the colonization of the host spleen by cells from the donor, cells capable of proliferation. When grafts are allowed to persist until the 12th to 14th day of incubation, *i.e.*, from 8 to 10 days after transplantation, a striking graft-*versus*-host reaction is observed. In more than 30 percent of 244 successful operations, there was a profound destruction of the entire vascular bed of the host embryo that resulted in hemorrhagic lesions. DeLanney (*6*) has been able to achieve a similar result by implanting a "rod" of adult salamander spleen into a "tunnel" in the somite region of salamander embryos at stages 25 to 28.

X irradiation of adult chicken spleen removes its ability to affect the homologous organ of the embryo. According to Mun, Kosin, and Sato (*116*), after irradiation at 205 to 810 r, splenic grafts retain their effectiveness; at higher doses, between 1230 and 1640 r, a significant decrease in effectiveness was observed, and at doses above 1845 r all activity was lost. These findings are, of course, consistent with the graft-*versus*-host hypothesis, which is supported also by studies using inbred lines of chickens. Adult spleens from two inbred lines of White Leghorn chickens, selected for leukosis susceptibility (S) and leukosis resistance (R), were implanted reciprocally and within lines to the chorio-allantoic membrane (*116*). If immune mechanisms are involved, one would expect a greater reaction between the two lines than within each line. Adult spleen grafts from donors of the S line affected spleens in embryos of the same line to a lesser extent than in embryos of the R line; spleen grafts from donors of the R line exerted a lesser effect in R hosts than in S hosts. When grafts were made from the parental strains to embryos of the F_1 hybrid, however, the results were unaccountably less consistent. Fragments of adult spleen from donors of the S line implanted into the F_1 (R × S) embryo produced an effect similar to that obtained following grafts of S-line spleens on R-line embryos. Conversely, spleens from R-line adults implanted into the F_1 (R × S) embryo produced an effect similar to the intraline combination. The authors noted also that grafts of spleens from adult S-line donors consistently affected the embryo spleen more strongly (regardless of the line of the embryo) than adult R-line grafts.

These findings agree essentially with those of Cock and Simonsen (*117*) who also used inbred White Leghorn lines. In the latter study, when blood from adult birds of an inbred line (I) was injected into newly hatched chicks of a cross between two highly inbred lines (C and I), the host livers and spleens were affected drastically. When F_1 blood was injected into F_1 chicks, only a slight effect was observed. The graft-*versus*-host reaction may be effected by a graft of any tissue that contains immunologically competent cells; it is not confined to those tissues characteristically considered to be part of the immune system, *e.g.*, bone marrow, lymph nodes, and spleen. For example, chorio-allantoic grafts of duodenum (*118*) and skin (*4*), which contain cells of the immune series, also may be effective.

FORMATION OF HUMORAL ANTIBODY BY ADULT CELLS IN THE EMBRYONIC ENVIRONMENT

The graft-*versus*-host reaction argues for the capacity of immunologically competent adult cells implanted in an embryonic environment to exert effects of the class usually described (for want of more precise information) as cellular immune responses. We may inquire next whether the embryonic environment will support the production of circulating antibodies by adult cells. Here we may distinguish three categories of experiment: (1) Adult cells are exposed to the antigen while they are still in the adult but are transferred before antibody synthesis is detected. (2) Unimmunized adult cells are removed from the adult and exposed to the antigen *in vitro*, after which they are implanted into the embryo. (3) Adult cells are confronted with the antigen for the first time in the embryonic environment. As in the foregoing section, attention will be directed chiefly to the chick, with such comparisons with other species as are pertinent. It will be noted that emphasis is placed on the inoculation of cells into the embryonic environment rather than on their inoculation into irradiated adults, for in the latter the interpretation may be obscured by partial recovery of the host's immune mechanisms.

Sibal and Olson (*10*) provided evidence of the production of antibodies in the chick embryo by adult spleen cells previously exposed to antigen *in vivo*. Spleens were removed 2 days after donor hens were injected with BSA; spleen-cell suspensions were placed on the chorio-allantoic membranes of 9- and 10-day embryos and antibody titers of the fluids from the transplants and adjacent areas of the membrane were determined after varying periods. Significant titers were observed within 4 to 7 days after inoculation. However, when 7 days or more elapsed between the antigenic stimulus and transfer to the membrane, antibodies were not detected. Nor were Sibal and Olson able to demonstrate antibody production when adult cells were exposed to antigen *in vitro* prior to inoculation. The latter results agree with findings of Dixon and Weigle (*119*) who found that cells removed from adult rabbits 17 to 21 days after a final injection of protein antigen did not produce antibodies after exposure to the antigen *in vitro* or when transferred to newborn rabbits simultaneously injected with antigen. It should be noted in passing that Sibal and Olson make no mention of deleterious effects of spleen cell inoculums on the host embryos; yet in examining their tables, it is apparent that the number of embryos surviving dropped drastically from the 2d to the 7th day after inoculation. In fact other experiments reveal that the graft-*versus*-host reaction may succeed in cells in which the production of circulating antibody fails; Van Doorenmaalen (*in 102*) has observed that after grafts of spleen previously sensitized with BSA pronounced graft-*versus*-host reactions may occur in embryos in which precipitins against BSA cannot be detected. To summarize: Adult cells previously sensitized with antigen *in vivo* clearly are capable of antibody production in the embryo. Demonstration of the antibody requires: (*a*) that the host be an embryo in which immunologic responsiveness is not yet initiated; (*b*) that the host be examined relatively soon after inoculation, before a graft-*versus*-host reaction predominates; and (*c*) that a highly sensitive technique be used to detect antibody.

In the second category, it has been noted that Dixon and Weigle (*119*) and Sibal and Olson (*10*) failed to detect antibody when adult cells were exposed to the antigen *in vitro* before they were inoculated into the embryo. (In the report by Sibal and Olson the incubation period was 30 minutes.) In order to assess the significance of these reports, we must treat first the question of the synthesis of antibody by cells *in vitro*. Cells from lymph nodes and spleens of immunized animals form antibodies *in vitro;* the first conclusive evidence is that of Fagraeus (*120*). Her findings have been verified repeatedly and have now been extended to the single cell (*121*, *122*). Although the production of antibodies by explanted cells from immunized animals is readily demonstrable, most attempts to *initiate* antibody formation *in vitro* have failed. It was 46 years before the first report by Carrel and Ingebrigtsen (*123*) was confirmed. Stevens and McKenna (*124*) found that rabbit spleen cells produced antibody when exposed *in vitro* to bovine γ-globulins, but only if the animal or spleen was pretreated with *S. typhi* endotoxin. Now, in an important preliminary communication, Fishman (*125*) has demonstrated the production of antibodies against T_2 bacteriophage and against hemocyanin in cultured lymph-node cells from normal rats. We may consider the T_2-antiT_2 system as an example. It was necessary that the virus be added first to a suspension of rat cells taken from a peritoneal exudate which was obtained 48 hours after the intraperitoneal injection of beef-infusion broth. These cells are largely macrophages. After being washed and packed, the macrophages exposed to the virus are homogenized and the homogenate passed through a Seitz filter. When the resultant cell-free filtrate is added to rat lymph-node cells *in vitro*, the latter are stimulated to produce antibodies (as revealed by phage neutralization) within 5 days at 37° C. Neither rat macrophages nor bacteriophage added to the cells singly is effective stimuli. In the bacteriophage-macrophage system, the rat macrophages cannot be replaced by rabbit macrophages or HeLa cells; they can be replaced, however, by a homogenate of rat macrophages.

Fishman's provocative findings present us with an opportunity to go beyond the tentative generalization of "teamwork" between the macrophage and the antibody-producing cell. Coons (*69*) and Wissler *et al.* (*70*) have reviewed the possibility that antibody formation involves the participation of a team of cells (*see also* p. 77). If Fishman's observation, that the requirement for intact macrophages can be replaced by a homogenate of those cells, proves to be a general phenomenon, then it appears entirely possible that the role of the macrophage may be clarified. The observations just described, however, make it unlikely that adult cells will produce antibody in the embryo following a brief exposure to antigen *in vitro* unless a procedure similar to that devised by Fishman is used.

But what of the third category, antibody production by adult cells exposed to antigen for the first time in the embryo? According to Trnka and Riha (*11*) antibody is formed by adult chicken-spleen cells injected *simultaneously with antigen* into either 18-day embryos or 2-day-old hatched chicks. Moreover, antibody production is better in the embryo than in the hatched chick, possibly because tolerance or partial tolerance of the spleen cells is induced that results in the absence of a host-*versus*-graft reaction in the younger animals. Ultimately, most of the animals die of a graft-*versus*-host reaction. Two antigens were used (singly): *Brucella suis* and BSA. Control animals received only spleen cells or only antigen. All 37 animals injected with *Brucella* plus spleen cells produced antibody; the embryos produced antibody with higher titers than did the hatched chicks. None of 26 birds injected with BSA plus spleen cells at 2 days post hatching produced antibody, but antibody was detected in 21 of 27 injected embryos. Coupled with the previous finding that spleen cells subjected to antigen *in vitro* before inoculation into the embryo do not form antibody, these experiments suggest, but do not prove, that the initial antigenic receptors (which appear to be absent *in vitro*) may be present in the chick embryo. They indicate clearly that with one refinement, namely, the inoculation of a pure line of lymphoid cells, devoid of macrophages, proof might be forthcoming. Evidence to be advanced in the following section suggests that the embryonic spleen is capable of receiving and "recording" an antigenic stimulus.

REACTIONS OF THE CHORIO-ALLANTOIC MEMBRANE TO SERIAL TRANSPLANTATION OF FRAGMENTS OF EXTRA-EMBRYONIC MEMBRANE AFTER INITIAL CONTACT WITH ADULT SPLEEN

The epithelial and mesenchymal cells of the chorio-allantoic membrane are labile, capable of keratinization (*126*) and striated muscle formation (*102*). DeLanney and Ebert (*127–129*) have followed cytologic changes in the chorio-allantoic membrane at closely timed intervals after implantation of homologous adult spleen. Both the epithelium of contact and the mesenchyme respond rapidly to the implant, the former by varying degrees of thickening, the latter by the formation of spindle cells and a shift toward myelogenesis. Intense membrane activity accompanied by proliferation and thickening can be demonstrated within 48 hours. This aspect of our program is largely a reappraisal of the pioneering studies of Danchakoff (*130*, *131*), as required by the graft-*versus*-host concept. The following comments are intended only as a synopsis of a detailed account now in preparation. Chorio-allantoic grafts of adult chicken spleen have been made on both the 7th and 9th days of incubation. After implantation at day 7, recoveries have been made, with few exceptions, at daily intervals through the 14th day of incubation, in addition to recoveries in the latter quarter of the incubation period. When grafts have been made on the 9th day, the principal recoveries have been made at days 12, 14, and 17. Here we will emphasize the cytology of the graft and adjacent chorio-allantoic membrane on days 8, 9, and 11 after implantation on day 7. At day 8, after residence of 24 hours on the membrane, most grafts are not incorporated fully. Regional differences appear in the graft; its perimeter is basophilic, composed in large part of mesenchyme cells having distinct, large nuclei. The central mass or "core" is eosinophilic (Dominici stain), and contains dense aggregates of small nuclei (according to Danchakoff, small lymphocytes). Examining the zone of junction of graft and membrane, one is impressed by three features:

(1) The union usually is marked by erosion of the chorionic epithelium. (2) "Tongues" of cells with small, dense nuclei can be seen invading the membrane. (3) Spindle cells appear in the membrane usually in apposition to the union of graft and membrane. The population of these cells is not large in number; we are inclined to argue that they are of graft origin, but cannot prove that we are correct. A few clusters of granulocytes may be seen also in the vicinity of contact which may be edematous. The events occurring during the 1st day continue during the 2d day after implantation; as a result the chorio-allantoic membrane surrounding the graft now differs strikingly from the normal membrane. The membrane is thickened, not as a result of edema, but as a result of the aggregation of cells. These dense aggregates are highly concentrated immediately under the graft. Aggregates of small lymphocytes (Danchakoff) are encircled by spindle cells.

Granulocytes may be identified, but they are not abundant. By day 11, however, the trend toward myelogenesis is pronounced, granulocytes being relatively common. Hemocytoblasts may be observed. At this time, also, the mucopolysaccharide content of the affected areas of the membrane is pronounced. Cysts, isolated pockets of degenerating cells, possibly of graft origin, are surrounded by giant cells. Keratinization as a major histologic feature is rare. We shall not pursue the cytologic changes beyond the 11th day at this time; processes already under way are heightened, but it appears that no new processes are initiated.

Previously, the graft-*versus*-host reaction has been transferred serially by intracoelomic and chorio-allantoic transplantation of embryonic host spleen after a primary graft of adult spleen (*see* p. 86). We can report here the serial transfer through three transplant generations of the graft-*versus*-host reaction by fragments of chorio-allantoic membrane in contact with a primary implant of adult spleen. The transplantation to the membrane of 7- or 9-day embryos of fragments of chorio-allantoic membrane, taken from reactive sites surrounding the original spleen implant, results in reactions in second and third generations, which must be described as intensified graft-*versus*-host reactions, characterized by extreme conditions of vascular breakdown. These grafts effect a rapid destruction of large areas of the chorio-allantoic membrane surrounding them. The contrast between the appearance of the membrane following a primary implant of spleen and a secondary graft of chroio-allantoic membrane is striking indeed. Within 4 days after implantation, the membrane of the host is moribund, marked by accumulated pyknotic nuclei and chromatin debris, as well as large areas which remain unstained with toluidine blue. Numerous aggregates of giant cells may be seen.

These findings, coupled with biochemical evidence yet to be described, have led the authors to postulate that the changes observed embrace two, and possibly three phenomena, *i.e.*, the effects of splenic grafts are more than a graft-*versus*-host reaction. The mechanisms of balance and interplay between these reactions are problematic. Out of a search for evidence for these interactions have come two findings of uncommon interest.

ANALYSIS OF PROGRESSIVE CHANGES IN EMBRYONIC CHICK SPLEEN AFTER EXTRA-EMBRYONIC GRAFTING OF HOMOLOGOUS ADULT SPLEEN

Transplantation of homologous adult spleen to the coelom or extra-embryonic membranes of the chick embryo evokes an increase in the myeloid elements of the host's spleen. The myeloid response is succeeded by the "graft-*versus*-host" reaction. Emphasis has been centered previously (*7*, *8*, *13*, *113–115*) on the terminal stages of the reaction following prolonged exposure to grafts (8–11 days post implantation). DeLanney and Ebert (*127–129*) have now focused attention on progressive changes in the host's spleen, with emphasis on the initial stages. Several thousand embryonic spleens, accumulated in earlier studies, have been examined, in addition to 758 spleens taken from recent experiments in which grafts were made to the chorio-allantois on the 7th day of incubation, with recovery at days 8, 9, 11, 12, 13, 15, 17, and 19. Since the first dramatic increase in weight of the host's spleen occurs on day 12, cytologic studies have been centered about this critical period. Normal and experimental spleens have been compared, employing principally the Dominici technique, Himes-Moriber triple stain, and toluidine blue, *p*H 4. The cytogenesis and histogenesis of the normal chick spleen have been described, as have the changes in the host spleen after homologous transplants (*129*). In normal development, although granulopoiesis may be detected infrequently as early as embryonic day 8 with granuloblasts observed in a significant minority through day 11, it is only at day 12 and later that granulopoiesis becomes a major event. In the experimental series, there is a pronounced shift toward granulopoiesis by day 11, followed by a spectrum of changes, not all of which are observed necessarily in each embryo: extreme granulopoiesis, accumulation of mucopolysaccharide, breakdown of the vascular bed, and necrotic and fibrotic foci.

IS THE EMBRYONIC CHICK SPLEEN ABLE TO RECEIVE A PRIMARY ANTIGENIC STIMULUS AND TO RESPOND TO A SUBSEQUENT CHALLENGE?

The spleen of the normal chick embryo, even during the latter quarter of the incubation period, appears to be incapable of a graft-*versus*-host reaction. When fragments of spleen from 17-day embryos are grafted to the chorio-allantoic membrane of 7- to 9-day hosts, the latter develop normally. Ebert reported a slight but significant increase in the size of the hosts' spleens; DeLanney and Simonsen, on the contrary, in a smaller number of experiments, recorded no effect whatsoever. These authors agree that there are no pathologic lesions in the spleens or other organs such as are found in the terminal stages after grafts of adult spleen. From the foregoing discussion, however, we may inquire whether the inability of the embryo-spleen cells to effect the reaction results from their *inability to receive an antigenic stimulus or from their inability to respond.* Let us consider the following simple experiments; a part of the findings are summarized in table 1, which is taken from (*102*): First, fragments of adult chicken heart were transplanted to the chorio-allantoic membrance of 7-, 9-, or 11-day hosts. It was known from numerous earlier experiments, and amply verified in the

Table 1.—Effect on the host embryo of chorio-allantoic grafts of 17-day embryonic spleen previously exposed to stimulus of adult heart grafts

Series	Age of host at operation (*days*)	Donor tissue	Age of host at recovery (*days*)	Number recovered / Number attempted	Mean weight of host spleens (*mg.*)	Range (*mg.*)
S–59–1	9	Sham	17	117/120	10. 2	7. 9–11. 3
S–59–2	9	Normal 17-day embryo spleen	17	59/60	12. 7	7. 4–17. 8
S–59–3	9	Unimmunized adult spleen	17	42/60	22. 9	8. 0–69. 0
S–59–4	9	Adult heart	17	59/75	9. 9	6. 1–12. 0
S–59–5	9	Adult heart	17	32/50	10. 3	7. 4–11. 7
S–59–6	11	Adult heart	17	25/40	8. 8	6. 3–13. 2
S–59–7	9	17-Day embryo spleen recovered in S–59–3	17	39/100	39. 6	11. 0–102. 0
S–59–8	9	17-Day embryo spleen recovered in S–59–4	17	41/57	16. 1	6. 0–44. 0
S–59–9	9	17-Day embryo spleen recovered in S–59–5	17	53/64	18. 8	7. 4–40. 0
S–59–10	9	17-Day embryo spleen recovered in S–59–6	17	21/48	12. 2	8. 8–40. 3

current series, that heart grafts are incapable of exerting a graft-*versus*-host reaction, unless perhaps the graft contained a "nest" of immunologically competent cells. Only 4 such exceptions are recorded in well over 600 heart grafts. At the 14th or 17th day, 7, 8, and 6 days after the initial grafts at 7, 9, or 11 days, respectively, two classes of second-set grafts were established in normal 7- or 9-day embryonic hosts. In the first class, fragments of 17-day spleen that were taken from embryos which had borne heart grafts for 8 or 6 days were transferred. It must be emphasized that these spleens were normal in appearance and size. Suitable controls, including sham operations, grafts of normal 17-day embryonic spleen, grafts of adult chicken spleen, and for comparison, grafts of embryonic spleen taken from recipients of first-set adult spleen grafts were used. The results are consistent, many of the second-set grafts exerting an effect on the host spleen. In contrast to a mean host-spleen weight of 12.7 mg. after grafts of normal 17-day spleens, the second-set grafts of spleen following a primary heart stimulus produced host-spleen weights averaging 16.1, 18.8, and 12.2 mg., respectively. It will be noted that in the last series the initial stimulus was 6 rather than 8 days. Of these host spleens, 9 presented the cystic condition which is characteristic of the graft-*versus*-host reaction.

The second group was composed of experiments similar in principle to those described on page 91. Fragments of chorio-allantoic membrane adjacent to the original heart grafts were transferred; the findings are less consistent. They do suggest that cells in the membrane have been sensitized, and are able in turn to have an impact on the secondary host spleen. Mean weights differ from the controls, but not strikingly. The most effective summary of these experiments (L.E.D. series XIV and XV) is the following: 15 of 55 spleens in series XIV and 16 of 77 in series XV exceeded the maximum weight attained by the appropriate controls of each series.

What interpretation is to be advanced for these preliminary findings? First, we must ask whether they can be explained on the basis of the existing phenomenon, the graft-*versus*-host reaction. Are the results based on the transfer of a small population of immunologically competent cells from the graft? It was mentioned earlier that in only 4 embryos out of many hundreds has a recognizable graft-*versus*-host reaction been observed after a graft of heart (ventricle). Is it possible, however, that in most heart grafts, the incidence of active cells of the immune series is very low, and requires the longer period afforded by transfer to a 2d embryo to exert their effects? This explanation is unlikely—but not ruled out—by the following facts: (1) The appearance and size of the host spleen after heart grafts is remarkably consistent with the pattern in the normal embryo. There is no suggestion of the proliferation of even a small population of donor cells or of a mild effect on the host liver or spleen. (2) The effect of a second-set host spleen (S–59, groups 8 to 10) is greater than that of a second-set fragment of chorio-allantoic membrane (L.E.D. XIV, XV), whereas the two effects are at least equal in magnitude in the graft-*versus*-host system.

These considerations lead to the tenuous conclusion that the findings cannot be explained on the basis of graft-*versus*-host reaction. Moreover, the results are not those which might have been expected from a theoretical consideration of actively acquired tolerance, namely, that if the graft influences the embryo's immune mechanisms, that influence should have been in the direction of tolerance. In theory, therefore, there is no obvious reason to expect that immunity would be provoked in the embryo. Perhaps the explanation is to be found in the following series of arguments: (1) Immunologic tolerance is specific with respect to isoantigens; thus tolerance would have been demonstrable only if the second-set (spleen) graft were made to an embryo of the same inbred line. Inasmuch as we have deliberately not used inbred chicks, we would expect, at best, partial tolerance for common antigens. [To consider an earlier argument (p. 78), are viable cells other than immunologically competent cells effective in evoking tolerance?] (2) By the same argument, however, since the stimuli for immunity and tolerance are the same, the only difference being the time at which the stimulus is administered, true second-set immunity would not be expected. (3) If these arguments are correct, we can ascribe the findings to a stimulus only of the first stage in the immune reaction, namely, the stage at which antigen is engulfed or fixed (it is difficult to employ a term that does not imply a mechanism) before the antigenic message reaches the responding cell. Thus the role of the first-set graft may parallel the role of the beef-infusion broth used by Fishman to evoke the production of macrophages, *i.e.*, it effects a mobilization of the initial receptors, a hypothesis readily subject to test.

SELECTIVE UPTAKE OF SUBCELLULAR FRACTIONS BY THE CHICK EMBRYO; MODIFICATION OF PATTERNS OF SYNTHESIS IN THE HOST

To view the findings of DeLanney and Ebert (*6–8*) within the framework of the observations of Billingham and Brent (*5*) and Simonsen (*9*), one cannot doubt that grafts of immunologically competent tissues react against their hosts, and lead to the formation of cysts and other pathologic lesions in their spleens and other organs and ultimately to death. Moreover, it is clear that cells leave the graft and enter the extra-embryonic membranes and the embryo proper—at least some of the cells residing in the homologous organ of the host. The extent and nature of this cellular contribution and its role remain to the authors largely a matter of conjecture, though Simonsen has argued that the increase in size of the host spleen is due only to the proliferation of adult cells populating the embryonic spleen, the proliferation being the result of antigenic stimulus.[4] The transplantation of tissues marked with tritium-labeled thymidine may be an important step in resolving the dilemma. Whether tissues transplanted to the embryo emit complex matter including intracellular particulates and large molecules which operate in regulating the synthesis of macromolecules in the host is another question. Can the modification of the patterns of synthesis in the host be achieved as a consequence of the predominant localization, not only of graft cells but also of particles or molecules, in the homologous organ of the host? Thought must be given also to the possibility of regulation indirectly by the localization in an organ of antibodies or products of immune reactions directed against the antigens in the original inoculum. In all these phenomena one fundamental theme recurs, namely, the question of the constant emission by normal cells of specific regulatory substances.

A full re-examination of this complex problem is beyond the scope of this article. We shall attempt to bring together our older data, the recently published findings of others, and research completed in our own laboratories and only now being prepared for presentation in detail. When adult cells are transplanted to the early embryo, a stimulation in the growth of homologous tissue is observed, exemplified by increased growth of the embryonic chick spleen after transplantation of a fragment of adult chicken spleen to the chorio-allantoic membrane of the 9-day host embryo. In the past we have used the expression "quantitatively organ-specific" to describe the reaction, for, of 11 donor tissues studied by Ebert, only 3 are capable of evoking the response; they are in order of decreasing effectiveness: spleen > thymus > liver. Evidence (*118*) indicates that grafts of small intestine also may be effective, as are skin grafts (*4*). Inasmuch as each of these organs may contain immunologically competent cells, it would appear more consistent to state that the reaction is tissue-specific, with the added stricture that it may be possible eventually to define it more precisely as cell-specific. Clearly the shift in the host spleen is toward myelogenesis (granulocytopoiesis). For the moment, however, we should emphasize that the quantitative organ specificity reflects the proportion of immunologically competent tissue it contains. The phenomenon is class-specific but not species-specific, adult mammalian spleen being ineffective, whereas fragments of spleen of other avian species do stimulate the growth of the chick spleen, though never to the same extent as the homologous tissue (*see* p. 86).

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[4] Recent pertinent experiments of Clarkson and Karnofsky must be noted. The following brief summary of findings now being prepared for publication is presented with the permission of Dr. Clarkson: The fate of suspensions of human and mouse leukemias and cancers, as well as normal cells when injected intravenously into the 10-day chick embryo, and their effect on the host have been studied. Generally, 500,000 to 5,000,000 cells are injected. In most experiments, 5 different ascites mouse leukemias caused significant splenic enlargement in the chick embryo, some spleens weighing up to 120 mg. in the 20-day embryo. Occasionally, with the same procedures, no splenomegaly occurred for reasons not understood. Normal or leukemia mouse splenic cells caused lesser, but still significant, splenomegaly. Neither human leukemias, acute and chronic, nor any of the numerous human or mouse tumors tested produced splenomegaly, irrespective of whether the tumor cells failed to grow, or grew rapidly and killed the chick because of metastases. Bioassay experiments, in which organ suspensions from chicks previously injected with mouse leukemia at 10 days are reinjected into mice, have shown that these suspensions are capable of inducing leukemia for at least as long as 10 days after hatching. By various labeling techniques, it has been conclusively demonstrated that active proliferation of mouse leukemia cells occurs in the chick embryo in at least 1 of the mouse leukemias. No evidence has been found that human leukemia cells proliferated in the chick embryos and some evidence indicates that they do not. The chicks harboring the mouse leukemia cells as a rule develop normally and show no ill effects. By means of fluorescent antibody studies, it has been shown that insufficient numbers of mouse cells are present to account for the splenomegaly. The simplest explanation for the splenomegaly is that the leukemia cells are producing a substance which causes splenic enlargement. It is possible that this postulated mechanism is also operative in the splenomegaly produced by homologous spleen cells or leukocytes in the chick embryo.

Grafts of adult chicken spleen, made to the coelom of the host chick embryo before the appearance of the host spleen, stimulate the host's spleen by the 10th day. The question was raised whether such a stimulated 10-day embryonic spleen might not in itself be an effective donor. Normally the 10-day embryonic spleen is ineffective. Grafts were made to 4-day embryos, which were permitted to develop until the 10th day, when the host spleens were harvested and used as the donor tissue for a second set of grafts, the experiment being carried through four transplant generations. The growth-promoting activity was not diluted by serial passage, but was maintained relatively constant. The simplest explanation of these observations is the colonization of the host spleen by whole cells from the donor, cells capable of reproduction. Although histologic analysis of the primitive host spleen gives no evidence for the transfer of large populations of adult cells, the transfer of a seed population of graft cells is probable. Adult cells do not proliferate extensively in the graft (*cf. 116*). Why then should they proliferate extensively in the splenic primordium? Simple "colonization" does not offer a complete explanation.

Studies with radioactive-labeled transplants showed that when the proteins of the donor tissue were radioactively tagged, labeled materials from the graft were localized predominantly in the homologous organ of the host. The hypothesis was advanced that macromolecular components released by the graft were localized selectively in the homologous organ; it was suggested that, although these macromolecules were not incorporated directly into the host proteins, the amino acids and peptides into which they were fragmented before incorporation never entered the general amino acid pool. This investigation, which embraced cytologic and autoradiographic studies and analyses of deoxyribonucleic acid in stimulated tissues, showed that the stimulation does not result from a massive transfer of intact cells (*8*, *13*, *80*, *81*). Again, it did not rule out the transfer of a seed population of viable cells.

Among the considerations that cannot be easily reconciled with the argument that viable cells stimulated by antigen to proliferate constitute the sole factor are: the effectiveness of embryonic grafts and the experiments of others, *e.g.*, Andres (*132*), which indicate the stimulatory effectiveness of frozen, thawed, triturated tissues. In fact, devitalization often has been shown to improve specificity and stimulatory effectiveness. In an effort to define more precisely the nature of the substances transported from graft to host tissue and the manner in which they act, tissues of adult chickens were labeled, as in earlier experiments, by the injection of methionine labeled with S^{35}. These organs were stored in the frozen state, after which they were homogenized, and a small amount of the homogenate was injected directly into the chorio-allantoic vessels of the 9-day embryo. The results of these experiments, in which labeled kidney and spleen homogenates were injected on either the 9th or 10th day of incubation with recovery after 24 hours, show that the exposure of the embryo to a very small quantity of tissue homogenate for only 24 hours results in a differential incorporation of the label in the homologous organ. The differential is not of the same order as that obtained with living transplants, acting over several days, but it is consistent and reproducible. The next step was the fractionation of the homogenate following standard (sucrose) procedures for the separation of nuclear, mitochondrial, microsomal, and supernatant fractions. The findings show clearly a selective localization only of the microsomal and supernatant fractions, and not of the nuclear and mitochondrial fractions. The properties of the microsomal and supernatant fractions which determine the specificity or which are essential to the localization are nondialyzable, and are destroyed by heating at 80° C. for 5 minutes, and by ashing. These experiments dealt with only one aspect of the problem, namely, predominant localization. They did not contribute to an understanding of how the homologous organ is stimulated. The small quantities of these subcellular fractions which were tolerated by the embryo do not result in a significant growth stimulation. It was suggested that a continuing supply of injected materials may be necessary to bridge the gap between the transplantation and injection experiments.

Although tissue-specific mechanisms other than those involved in the immune response appeared to be involved, the graft-*versus*-host reaction rendered the analysis difficult. Neither experiments using as donors and hosts members of highly inbred lines of chickens (*116*, *117*) nor experiments with the use of filters which preclude cellular transfer from graft to host have been fully convincing. In the latter experiments splenic grafts encased in Millipore filters evoked a significant increase in the size of the homologous organ of the host. The increase was of the order of 30 percent, significant, but far less than the several hundred percent increase attained by grafts in the absence of any filter.

At this point, Ebert (*8*, *50*) questioned whether, in view of the ultimate destruction of the host tissues, the use of organs such as spleen, for further tests of the hypothesis of tissue-specific regulation of differentiation and growth, was advisable. Although his note of skepticism may prove ultimately to be well taken, more recent developments suggest that it may have been premature. Croisille (*133*) has reported that the intravenous inoculation, in a series of 3 injections, of a cell-free extract of adult chicken spleen into the chick embryo, beginning on the 3d day of incubation, results in a significant increase of 25 percent in the size of the host spleen. The recipients were recovered at 12, 14, 16, 17, and 18 days of incubation; no evidence of cysts or lesions related to the "graft-*versus*-host" reaction was reported. Similarly extracts of liver stimulate embryonic liver growth when injected *in ovo*. Croisille reports, however, that attempts to demonstrate similar effects *in vitro* failed. Van Haeften (*134*) implanted cell-free homogenates of adult spleen, thymus, and striated muscle on the chorio-allantoic membranes of 9-day embryonic hosts. The spleen homogenate provoked a strong myeloid reaction in the chorio-allantoic stroma as well as marked hypertrophy of the host spleen. Histologically, the enlarged spleens were normal. After inoculation of spleen homogenate, there is also an increase in all cell types in the circulating blood; after thymus homogenate, lymphocytosis, and eosinophilia.

These findings prompted us to re-examine our own earlier results, which had demonstrated predominant localization, but not stimulation, with microsomal preparations. It seemed particularly important since Croisille's data on stimulation by extracts paralleled quantitatively our own findings with membrane filters. It would not be rewarding to retrace all the thinking behind the decision, but it was decided to examine the efficacy of a fraction prepared according to the procedure described by Moloney (*135*) for purification of the Rous sarcoma virus. It will suffice to say that one of us has been using the technique (*102*) in experiments in which combinations of the Rous sarcoma virus and a tissue-specific microsomal fraction have been shown to be effective homeogenetic inductors. In the experiments to be described, the virus was not included. The technique commonly applied for virus purification was used for the extraction of spleen and other organs—preliminary hydrolysis of the tissues by hyaluronidase, formation of a protein-protein complex using protamine sulfate, followed by tryptic digestion, and separation of the microsomal fraction by differential centrifugation (*135*, pp. 878–882). Tissue fractions obtained by this procedure were administered to the embryo by two routes, intravenous injection (*136*) and inoculation directly to the chorio-allantoic membrane. The latter route is less effective. The inoculation of 0.1 to 0.15 ml. of a splenic microsomal fraction on the 9th or 11th day resulted in a highly significant increase in the weight of the host spleen (table 2), as compared with the sham-operated controls and with embryos receiving microsomal fractions from other organs (*see 102* for details).

Although we are mindful of Lord Acton's cogent remark that the worst use of theory is to make man insensible to fact, we will attempt to view these findings in relation to the pattern that has emerged. The mecha-

Table 2.—Mean weights of spleens of 17-day chick embryos after inoculation of "microsomal fractions" of adult organs

Series	Age at inoculation (*days*)		Source of inoculum							
			Sham	Spleen	Liver	Kidney	Heart	Brain	Ovary	Testis
MF 59, 1 + 2	9	1) Number of embryos	51	50	48	50	30	x	x	x
		2) Mean weight (mg.)	11. 1	19. 6	10. 9	9. 9	10. 7	x	x	x
		3) Range (mg.)	8. 9–14. 0	13. 1–28. 4	7. 6–12. 9	8. 9–13. 6	8. 0–13. 6	x	x	x
MF 59, 3 + 5	9	1	53	52	56	51	x	23	x	x
		2	10. 2	14. 1	13. 0	10. 7	x	10. 9	x	x
		3	8. 2–13. 1	11. 3–19. 3	8. 3–14. 9	9. 3–15. 1	x	8. 6–17. 1	x	x
MF 59, 6	9	1	33	67	31	40	x	x	25	25
		2	10. 8	16. 7	13. 9	11. 0	x	x	11. 1	10. 7
		3	9. 0–13. 3	15. 6–19. 6	9. 1–18. 2	8. 4–13. 0	x	x	7. 2–15. 0	6–9–14. 6
MF 59, 9	11	1	25	31	50	x	25	24	x	x
		2	9. 7	14. 3	12. 2	x	9. 6	10. 8	x	x
		3	7. 6–14. 3	10. 9–16. 3	8. 9–16. 1	x	8. 7–11. 3	8. 0–14. 1	x	x
MF 59, 11	11	1	12	29	25	30	26	25	x	x
		2	10. 3	13. 9	11. 9	8. 9	11. 1	10. 0	x	x
		3	8. 9–11. 0	11. 1–18. 7	11. 0–14. 6	6. 1–13. 6	6. 7–13. 4	8. 3–12. 7	x	x
MF 59, 13	11	1	12	33	26	29	x	x	20	18
		2	10. 7	15. 2	12. 4	10. 1	x	x	9. 4	10. 9
		3	9. 3–12. 1	11. 9–20. 1	9. 3–16. 8	8. 3–11. 6	x	x	8. 6–10. 2	8. 1–13. 1

nisms underlying these phenomena may differ widely; for this reason it would be premature, and probably unwise, to attempt a synthesis. We intend, therefore, only to enumerate and categorize those reactions which appear to fall within a broadly conceived conceptual framework.

Burnet's (*137*) clonal selection hypothesis of antibody production maintains that at birth there exist sufficient clones of mesenchyme cells to produce antibody globulin which is reactive against any type of foreign antigenic determinant. (Clones capable of reacting against autologous components must have been eliminated.) Burnet says "Two assumptions are needed: (1) that mesenchymal cells are highly mutable in respect to detail of the antibody globulin they produce, and (2) that some mesenchymal cells of the antibody-producing clones carry antibody-like sites which on reaction with the corresponding antigenic determinant stimulate the cell to proliferate and that the better the 'fit' between the antigenic determinant and the antibody receptor, the stronger the proliferative stimulus." Let us examine the several phenomena we have described with reference to these assumptions. First, the graft-*versus*-host reaction: Undoubtedly an immune reaction results in the destruction of the host. It is assumed, but not regarded by all as proved, that the increase in the host spleen results solely from *proliferation of graft cells stimulated by host antigens.* Until appropriate labeling methods are used, two notes of caution must be expressed. First, if the principal cells proliferating are those of the donor, what mechanisms tend to limit their proliferative activity to the myeloid centers of the host? Why does proliferation not occur extensively in the graft itself? Second, the evidence favoring donor-cell proliferation has been indirect, *viz.*, the ability to transfer graft-*versus*-host activity from embryo to embryo. The experiments described herein show that *this reaction can be transferred by host-spleen grafts after a stimulus from donor tissue, the heart, that is not immunologically competent.* This reaction casts doubt on the former interpretation. The possibility clearly exists that the initial mobilization phase of a host-*versus*-graft reaction may occur in the embryo. Here again an objection must be raised, namely, that although the host spleen may "receive" an antigenic message from a heart graft, its cells do not undergo proliferation. For proliferation, the stimulus of spleen (or a related tissue) is required. Moreover, it is clear that the stimulus for proliferation may be in the form of a subcellular particulate; viable cells are not required. Although the mechanism of action is problematic, the ability of spleen microsomes to stimulate γ-globulin production also must be cited. We revert to the idea of continuous and reciprocal exchange between the tissues of the organism, both in the embryo and adult, and between the host and an implanted tissue. This idea is not new; it is inherent in the theoretical discussions by Weiss (*138*, *139*) and is stated explicitly in a stimulating paper by Medawar (*140*).

These experiments could be interpreted on the basis of the concept of feed-back equilibrium (regulation of the differentiation and growth of a tissue by its own products), for they do suggest a balance between tissues of different physiologic age (*138*, *139*, *141*, *142;* reviewed by Paschkis, *143*). We fear, however, that such an interpretation might be misleading. Despite the recent contributions of Tumanishvili (*144*) and others (*see 143*), we believe that until proved otherwise, findings with immunologically competent tissue fractions must be viewed apart from generalizations regarding tissue-specific growth regulation.

If, in considering the effectiveness of spleen microsomes in stimulating proliferation in the host spleen and the production of γ-globulin, we ascribe to them only the properties of an antigenic stimulus, we must return to the enigmatic question, why spleen or related microsomes? Equally enigmatic is the question of the role of spleen (and similar) cells in relation to the mechanism of tolerance. Humphrey and Haldane have suggested (summarized, *4*) that embryos are able to metabolize molecules which in the adult are not metabolized but are antigenic. According to these views, the embryo would be able to metabolize and reutilize its own products; foreign materials introduced during the embryonic period would be treated similarly. It may be argued that during embryonic life a foreign molecule induces an enzyme which is specific for its own catabolism, an enzyme maintained at least as long as traces of substrate remain (*cf. 118*). It must follow that the capacity for induction of this class of enzymes is lost at maturation. It should be possible then to demonstrate that the injection of a foreign molecule, *e.g.*, a protein, during embryonic life would result in an induced enzyme, whereas the same protein in the adult would induce an antibody. Conversely, we may speculate that molecular species that lack antigenicity or are poor antigens should induce an enzyme even in the adult, *e.g.*, the injection of DNA or RNA should induce DNase or RNase.

We may add the additional argument, which appears entirely reasonable, that the aforementioned modification of protein metabolism, *viz.*, the breakdown of proteins, the reutilization of protein intermediates, induced-enzyme and antibody synthesis take place in the lymph nodes, spleen, and related centers of immunologic response. In doing so, we focus attention logically on the microsomal particles which have been implicated as the major sites of protein synthesis within the cells of a variety of tissues (reviewed, *8*, *145*, *146*). Schweet, Lamfrom, and Allen (*145*) have described the synthesis of soluble protein, mainly hemoglobin, in a system containing microsomal particles from rabbit reticulocytes plus soluble enzymes. Fully comparable evidence is not available for antibody formation, though Kern, Helmreich, and Eisen (*146*) have shown the presence of antibody activity on particles that have the sedimentation characteristics of microsomes. The activity was induced in lymph nodes which drain sites of injection of 2,4-dinitrophenyl-bovine γ-globulin into guinea pigs, and was specific for the DNP group. The presence of the antibody cannot, of course, be taken as evidence of synthesis of antibodies on the microsomes. As the authors make clear, their studies are equally consistent with the synthesis of antibodies elsewhere and their translocation to microsomes. The unusual affinities of cells of lymph nodes and spleen, their possible role in actively acquired tolerance, the predominant localization of splenic particulates in the embryonic host spleen after injection with a resultant increase in growth of the homologous organ, and the stimulation of γ-globulin production by injected microsomes—all of these phenomena—appear to find a common focus in the microsomes. Whether this pattern is meaningful remains to be determined (*13*, *80*, *147–151*).

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The Immune Response and the Homograft Reaction [1,2]

HENRY J. WINN,[3] *Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine*

The rapid growth and development of the study of tissue transplantation in recent years is reflected in the appearance of a number of reviews covering both immunologic and genetic aspects of the problem (*1–8*). Some of these reviews have been extensive in their coverage and have presented clearly and exhaustively the experimental evidence that has established the homograft reaction as an immunologic phenomenon initiated by antigenic differences between transplant and host (*1–4*). It is not necessary to preface a review of our subject with a restatement of this evidence, and I shall proceed to a discussion of an aspect of the immunology of homotransplantation, which, with the exception of a review by Gorer (*1*), has been treated only superficially. This aspect is the mechanism of graft breakdown, particularly as it involves the reactions that have been shown to operate in specifically acquired resistance to infectious diseases and in those allergic phenomena which are known to have an immunologic basis.

Immune reactions in general can be mediated by serum or by cells, and the relative importance of these two components is a matter of controversy at the present time, particularly with respect to graft rejection. Although it is quite possible that serum antibody and specifically activated cells are intimately related and may at times act together in causing rejection of homografts, it will be convenient to discuss these two immune substances independently.

In those immune or allergic reactions that can be transferred by cells but not by serum, the nature of the active substance is unknown, but virtually all investigators in this field have preferred to speak of it as an antibody, commonly as cellular or cell-bound antibody. To avoid confusion, the term *antibody,* when used to designate the classical serum-globulin variety, is often qualified by some more descriptive term, *e.g., serum antibody.* The terms *humoral, circulating,* and *soluble* have also been used to describe serum antibody. The term *circulating* is inappropriate, since it is certain that cellular or cell-bound antibodies are also carried through the blood stream. As more is learned of cellular antibody, some of the other descriptive terms may become obsolete, but presently they appear satisfactory for distinguishing between the two types of immune substances.

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[1] Presented at a symposium on normal and abnormal differentiation and development, held in celebration of the 30th anniversary of the Roscoe B. Jackson Memorial Laboratory, June 15–17, 1959, at Bar Harbor, Maine.

[2] This work was supported by research grants C-1329 and C-3578 from the National Cancer Institute, National Institutes of Health, Public Health Service.

[3] I wish to thank Dr. Nathan Kaliss for his critical review of this manuscript.

THE ROLE OF SERUM ANTIBODY

The present controversy and confusion regarding the role of serum antibody in the destruction of homografts stems from three main sources: (1) There has been, for rather obscure reasons, a reluctance on the part of most investigators to consider the possibility that more than one type of immune mechanism may be involved in the "homograft reaction." This attitude is difficult to understand in view of the diversity of immune mechanisms that have been observed in studies on bacterial immunity and hypersensitivity, and it has led to a tendency to generalize in conclusions based on experiments with grafts of a single-tissue type. In this connection it is interesting to recall the striking difference in sensitivity to complement that is observed between antibody-sensitized gram-positive and gram-negative bacterial cells.

(2) There is a widespread notion that if antibodies are important they should be cytotoxic *per se.* This is a rather serious misconception and has led to an unnecessary expenditure of time and materials. There is, as far as I am aware, no instance in which antibody alone has been shown to be cytotoxic. This statement holds for bacteria as well as for cells of various animal and plant tissues. Whenever antibody has been involved in the specific destruction of cells, critical investigation has implicated the participation of complement or phagocytes. (In hypersensitive reactions there is often a good deal of nonspecific tissue destruction which may, of course, be independent of complement and phagocytes, but it is certainly not due to the direct action of antibody. Moreover, a good deal of recent work has pointed to complement as an important mediator of hypersensitive reactions.) A variety of microorganisms can be grown *in vitro* in the presence of specific antiserum just as readily as in normal serum (*9*). Indeed, Braun (*10*) has recently investigated cultures in which immune serums can induce population changes among bacterial cells grown *in vitro*, and he has been able to show that the selection against smooth variants is based, not on a direct cytotoxicity of antibody, but on the anoxic conditions that result from agglutination of the smooth-phase organisms into large clumps. If aeration or mechanical agitation is practiced, the selection is no longer operative. Also, many gram-negative organisms may be grown quite well *in vitro* in media containing potent immune serum, if complement is not added.

(3) As Gorer (*1*) has previously pointed out, there is prevalent a naive assumption that, if antibody is involved in homograft rejection, it ought to be present in large amounts and at all times in the serums of animals that have rejected even a single tissue graft. The fallacy of such an assumption is, of course, only too obvious to immunologists who have become accustomed to the use of long series of injections for provoking good, measurable antibody responses. Moreover, much transplantation work is carried out in mice, a species whose antibody is

characterized by a relatively short half life. This will tend to make the time of testing for antibody in mice more critical than in many other species. Furthermore, there are numerous instances in which a high degree of specific resistance has been observed in patients or experimental animals whose serums were virtually devoid of demonstrable antibody. Sometimes this was due to the presence of nonprecipitating or blocking antibody; in other instances it merely reflected the ability of the immune mechanism to make a rapid anamnestic or "booster" response to the challenge of microbes or toxin. The possibility that non-antibody mechanisms were involved must also be considered but in many instances this could be excluded. The point I wish to make is that an animal may display a high degree of specific resistance in the absence of an appreciable level of serum antibody simply because it is in a position to make a good secondary response. Failure to achieve transfer of passive immunity with the serum of such a specifically resistant animal would obviously not constitute proof of a non-antibody mechanism of protection. Also invalid is the argument, recently advanced (*11*), that passively raising the titer of serum antibody to the level of a normally immunized animal should produce transplantation immunity, if serum antibody is to be considered important in the homograft reaction. This argument not only ignores the importance of the booster effect in actively immune animals but also fails to consider that the serum antibody in the latter group is constantly being renewed, whereas, in passively immunized animals, there is a rapid decrease in antibody concentration, due to normal metabolic activity. These considerations are particularly important in transplantation immunity since the destruction of homografts usually takes place over several days. That the argument has been raised at all is surprising in view of the fact that successful transfer of transplantation immunity by means of cells has in the past required a ratio of 4 or more donors for each host. One could hardly describe the recipients in such experiments as having been treated with normal physiologic amounts of lymphoid tissue.

Turning now to the actual experimental data bearing on the importance of serum antibody in the homograft reaction, we may begin with the early observations of Gorer, who first clearly demonstrated the appearance of an antibody that had been provoked by a homograft (*12*). Initially this antibody was demonstrated as a hemagglutinin, specific for the red cells of the graft donor or, since inbred mice had been used in this work, for red cells of all animals of the donor strain. The homografts used by Gorer were of a leukemia tumor, and it was subsequently shown that suspensions of cells prepared from this tissue could be specifically neutralized by the serums of mice which had been immunized by a series of tumor grafts. The technique used in this work consisted of mixing cells and serum *in vitro* and injecting the mixture into mice of the strain of origin of the tumor. Successful neutralization resulted in delayed or complete inhibition of tumor growth. This has subsequently been referred to as a neutralization technique, and the initial findings obtained with it have been observed with certain other mouse tissues (*13–15*) as well as with tumors in other species (*16*, *17*). In later studies Gorer and Amos (*18*) and Amos and Day (*19*) demonstrated the passive transfer of homograft immunity to leukemia tumors by means of serum.

We have recently conducted an extensive investigation of the mechanism and extent of applicability of the neutralization technique (*20*, *21*) and I should like to present some of the results and implications of our work before discussing those reports in which allegedly immune serums have been found lacking in neutralizing or protective properties. Table 1 contains the results of a typical neutralization test in which various numbers of cells of the 6C3HED lymphoma were mixed with 1.0-ml. portions of normal serum or C57BL/6 anti-6C3HED serum in a total volume of 3.0 ml. This mixture was injected into C3H mice at a dose of 0.2 ml. per mouse. The immune serum was prepared by a series of 6 subcutaneous grafts of tumor given over a period of 2 months. (This tumor invariably regresses in C57BL/6 mice.) The serum-cell mixtures were prepared at 4° C. and were injected into the hosts immediately after mixing. Similar tests were carried out with doubling dilutions of antiserum, and some evidence of neutralization could be observed even when dilutions of 1:1280 were employed. Curiously, it was observed that as the cell dose was increased, a higher ratio of antiserum to cells was necessary to achieve a given level of neutralization. This was shown to be due not to the increase in the total number of cells tested, but to the increase in the size of the inoculums received by individual mice. In other words, a mixture containing a high concentration of cells in antiserum might be thought to contain few if any viable cells if each of the hosts received 0.01 ml. of the suspension. If, however, this dose was increased to 0.1 ml., the results would indicate that virtually no neutralization had been accomplished. It was subsequently shown that the antiserum-cell mixtures contained as many viable cells as normal serum-cell mixtures, and that the actual destruction of the cells took place *after* the suspensions were injected into the mice. Successful neutralization required, then, the participation of the hosts, presumably in the form of contributing complement. In support of this contention it was reported that a striking enhancement of neutralization could be achieved by injecting the prospective hosts of the antiserum-cell mixtures intraperitoneally with guinea-pig complement. This treatment had no effect on the fate of grafts of tumor cells mixed with normal serum. Alternatively, complement could be added to the antiserum-cell mixtures *in vitro*, in which case the degree of neutralization was determined by the concentration of complement and the time of incubation. With weak or dilute antiserums, periods of incubation up to 6 hours were required for maximum neutralization. In the absence of complement, incubation *in vitro* had no effect on the outcome of neutralization tests. In a subsequent (unpublished) experiment we were able to show that tumor cells, which had been added to antiserum at 4° C. and then immediately spun down in a centrifuge and separated from the supernatant serum, had become as sensitive to complement as cells that had been incubated in antiserum for several hours.

TABLE 1.—Neutralization of lymphoma 6C3HED by C57BL/6 anti-6C3HED serum

Number of cells injected per mouse	Percent of mice dying with tumor*	
	Cells mixed with normal serum	Cells mixed with immune serum
2.5×10^6	100	100
0.5×10^6	100	10
0.1×10^6	100	0
20,000	100	0
4,000	100	0

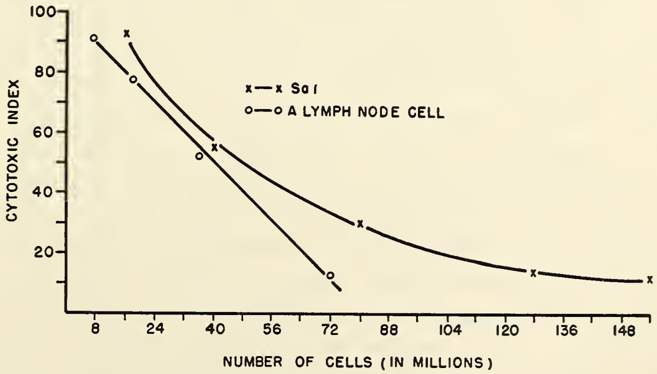
*Twenty mice in each group.

Our conception of the mechanism of the neutralization reaction is that the tumor cells combine with antibody almost immediately. The binding and subsequent cytotoxic action of the complement takes place over a period determined by the relative concentrations of the various reactants. This is not essentially different from the picture of immune hemolysis drawn by Mayer *et al.* (*22*). We do not, however, have any information on the importance of antibody transfer from cell to cell, which Mayer has described for red-cell lysis. The nature of the action of complement on the sensitized tumor cells is, of course, quite unknown, but it seems likely that it will not be too different from the action of complement in immune hemolysis, a phenomenon being intensively studied in a number of laboratories. When mice are injected with antibody-sensitized tumor cells that have not been exposed to complement *in vitro*, the outcome of the test depends on the concentration of host complement and the rapidity with which it reaches the injected cells, which are evidently capable of multiplying and producing cells that are not sensitized. Since antibody is not synthesized in compatible hosts, these unsensitized cells will grow progressively, which would lead one to conclude that the original mixture was not neutralized. Some transfer of antibody is to be expected, but when very large inoculums are used, a point will be reached where host complement will not suffice and tumors will invariably result from such implants. This critical role of complement may be exaggerated in the mouse, an animal notoriously low in several components of complement, but isoimmune reactions are far less efficient in the use of complement than reactions involving heteroantibody, a point recently discussed by Goodman (*23*). In actively immunized "foreign" hosts, the concentration of complement is less critical, for here both antibody and complement are being continually synthesized, and, judging from the reported half life of mouse antibody (2 days) and Osler's demonstration of the rapid recovery of complement levels in depleted animals (*24*), both substances are turning over rapidly, a situation that is ideal for destroying large numbers of cells. In passive immunity experiments, the transferred antibody will likely become exhausted before active formation of appreciable antibody levels, and again the level of host complement will largely influence the outcome of the experiment. Because the action of complement is proportional to the concentration of antibody over a wide range of concentrations, it is best to use antiserums taken from animals that have received a rather large number of grafts given over a period of several weeks.

Most of our early work on the neutralization technique had been carried out with the 6C3HED lymphoma. It was important to determine the degree to which the reactions of this tumor to serum antibody and complement could be considered typical of homografts of other mouse tumors. Twenty-five different types of antiserum were prepared from groups of mice that had received a series of 6 to 14 grafts of normal or neoplastic cells. These serums were then used in a series of neutralization tests of the type described. Two C3H lymphomas, a strain A lymphoma, a C57BL/10 lymphoma, 3 AKR lymphomas, and a C57BL/6 myelogenous leukosis were all found to be highly sensitive to antibody and complement; a strain A fibrosarcoma in both solid and ascites form, a B/10LP rhabdomyosarcoma, 2 C3H mammary carcinomas, and 3 strain A tumors originally diagnosed as a neuroblastoma, a spindle-cell carcinoma, and a salivary-gland tumor, respectively, were highly resistant to the cytotoxic effects of antibody and complement. Small numbers of the strain A sarcoma were adversely affected by one antiserum, but this activity was not augmented by the addition of large amounts of complement *in vitro* or *in vivo*. In the antibody-resistant tumors, additional tests disclosed that these tumors had stimulated the formation of antibodies and could react with and absorb such antibodies. Their resistance was clearly not due to the fact that their reactive sites were inaccessible to the antibody.

One antiserum prepared in C57BL/10 mice against SaI, a strain A fibrosarcoma, had no effect on this tumor in the solid or ascites form. It did, however, react with and neutralize a strain A lymphoma (*21*). Furthermore, as shown in text-figure 1, this antiserum had a high titer of cytotoxic antibodies for lymph-node cells as determined by the cytotoxic test *in vitro* first described by Pappenheimer and recently modified by Gorer and O'Gorman (*25*). SaI ascites tumor cells were completely resistant to this antiserum when they were tested simultaneously with the lymph-node cells. Portions of this antiserum were absorbed with known numbers of strain A lymph-node cells, SaI ascites tumor cells, or C57BL/10 lymph-node cells, and then retested against strain A lymph-node cells. The results of this experiment (text-fig. 1) showed clearly that the tumor cells could combine with the isoantibody, despite the fact that such sensitized cells remained viable in high concentrations of complement. On the other hand, the tumor cells were readily destroyed by complement when they were tested with an antiserum prepared in rabbits against tumor-cell fractions. The tumor cells in text-figure 1 are less efficient than lymph-node cells in absorbing antibody. Furthermore, the tumor cells are larger than lymph-node cells and, when the diameters of both cell types are measured and surface areas calculated on the assumption that the cells are spherical, the concentration of reactive sites on the SaI cells turns out to be approximately 4 percent of that on the lymphoid cells. This could conceivably greatly reduce the ability of the tumor cell-antibody complex to fix complement, and thus make it impossible for the latter substance to accumulate at the surface of the cell in concentrations sufficient to become toxic. In this connection it should be pointed out that the destruction of cells by antibody and complement does not require that the antibody react with vital or key cellular components. Red cells and bacteria that have been allowed to absorb various soluble antigens onto their surfaces may be readily lysed by complement and antibody specific for the absorbed antigens (*26*, *27*). Similarly, chicken cells that have been sensitized with PR8 influenza virus are lysed by complement in the presence of specific virus antibody (*28*). Apparently, cytotoxicity depends on the occurrence in the immediate vicinity of the cell of an antigen-antibody reaction capable of binding some minimal amount of complement. With cell isoantigens, therefore, the distribution of reactive sites and the total number of sites per cell would be critical in determining a cell's sensitivity to complement.

Our work, then, with the tumor-cell neutralization technique has enabled us to divide a number of transplantable tumors into two groups on the basis of their sensitivity to the cytotoxicity of humoral antibody and complement. That this division separates lymphoid and nonlymphoid tumors is no longer surprising, for the use of grafting and cytotoxic techniques *in vitro* is gradually revealing a similar division among normal tissues. Sensitivity to humoral antibody appears to be highly



TEXT-FIGURE 1.—Cytotoxic index of C57BL/10 anti-SaI serum for strain A lymph-node cells after absorption of serum with various numbers of SaI cells or strain A lymph-node cells. C57BL/10 lymph-node cells were completely ineffective in removing antibody in the dose range studied.

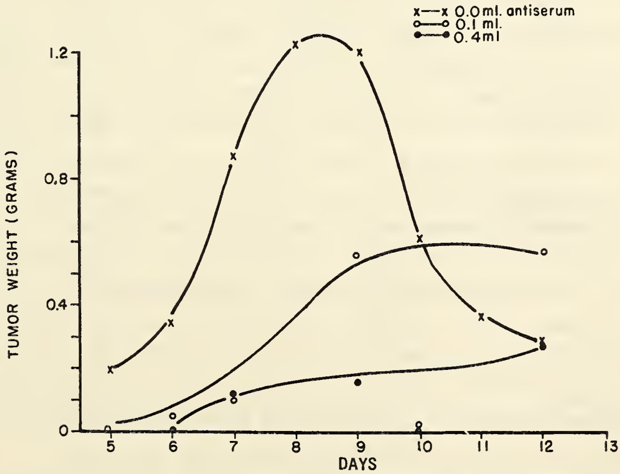
characteristic of lymphoid tissues, both normal and neoplastic. We have found, however, that thymocytes are completely resistant to isoantiserums (*29*), and Gorer has reported that a certain percent of bone marrow cells remain viable in the presence of specific antibody and complement (*8*).

The difference in sensitivity between thymocytes and lymph-node cells is paralleled by quantitative differences in the abilities of the two cell types to absorb antibody and to fix complement, the sensitive lymph-node cells being much more efficient in both activities. Cells of normal nonlymphoid tissues are extremely difficult to test for antibody sensitivity *in vitro* or by neutralization tests, but, as we shall discuss later, the available data from homograft studies strongly suggest that such tissues are insensitive to humoral antibody. However, resistance to the cytotoxicity of isoantibody and complement is not a property of the so-called "solid" tissues alone. This is most clearly seen in the resistance of cells of the ascites form of many nonlymphoid tumors.

The neutralization technique has often been criticized because the cells and antibody are brought together *in vitro.* This does, perhaps, provide more efficient contact between cells, antibody, and complement than is likely to be permitted in an established tumor graft, but, as we have pointed out on another occasion, this technique is not presumed to yield results that are in strict quantitative proportion to the effects of antibody in active transplantation immunity. It is designed primarily to test the sensitivity of cells to antibody, and because the sensitized cells are injected into mice of the strain of origin of the tissue being tested, the cells and serum must react first *in vitro.* It has been shown, moreover, that neutralization can be demonstrated even when the serum-cell suspensions are grafted immediately after they are mixed. There is no need to incubate them for prolonged periods, the principal reason for the step *in vitro* being to avoid absorption of the antibodies by the tissues of the host. Passive immunity experiments more closely simulate the conditions of active homograft rejection but have definite limitations: (1) They require much larger amounts of antiserum, a factor of considerable logistic importance where mice are involved. (2) The technique is restricted to those tumors that routinely produce good measurable growth in mice of incompatible strains, and even in these cases it is necessary to use inoculums containing very large numbers of cells. (3) Since the destruction of a homograft proceeds over several days, it is difficult to distinguish between the effects of the introduced serum and the developing active response of the incompatible host. (4) The neutralization technique has been more important in showing that the resistance of many nonlymphoid tumors to isoimmune serums is not due primarily to the inability of the antibodies to reach the tumor cells but to the distribution or form of the reactive antigenic sites on the surface of the resistant cells.

These comments on the technique of passive immunity to homografts are cited only to point out the reasons for using neutralization tests in many studies, and I would not like to leave the impression that passive immunity studies are unreliable or unimportant. We have, as a matter of fact, carried out passive immunity studies with all the tumors that we investigated by the neutralization technique. Tumors found susceptible to antibody by the neutralization technique were invariably found to be susceptible by the passive-immunity technique. A typical example of such a reaction is shown in text-figure 2. A large number of control and passively immunized animals were implanted with tumor at a dose of 7.5 × 10^6 cells per mouse. Groups of mice (20 for controls and 10 for each serum dose) were killed at various times after grafting, and the tumors were removed and weighed. The passively immunized animals responded to tumor implantation in one of two ways: Either there was complete absence of palpable growth, or a temporary delay in growth followed by the development of quite large tumors that regressed a few days later than tumors in control animals. The results appear to be somewhat erratic, *e.g.*, on day 10 the tumor weights were very much smaller than on days 9 and 12. This was due to the selection, by chance, on day 10, of only mice that had responded with little or no tumor growth. Mice that had been actively immunized with leukemia tumors never developed palpable growths when they were rechallenged with the specific tumors.

When the nonleukemia tumors were tested, it was found that in general they grew far better in passively immunized mice than in controls. In some instances this resulted in situations in which many or all of the antiserum-treated mice died with progressively growing tumors. This phenomenon has previously been described and studied in detail by

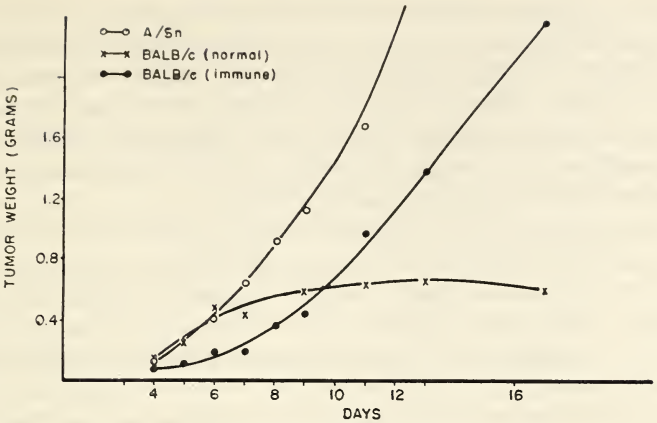


TEXT-FIGURE 2.—Growth of lymphoma 6C3HED in untreated C57BL/10 mice and in mice receiving intraperitoneal injections of C57BL/10 anti-6C3HED serum.

Snell (*5*) and Kaliss (*7*) in this laboratory, and elsewhere by Casey (*30*). It has been described variously as an XYZ effect, enhancement, and immunological enhancement. The latter term appears to be more suitable than the others and should replace them in current literature. Actually, the earlier studies on this phenomenon were carried out by injecting the prospective tumor hosts with preparations of killed tissues. The enhancement of tumor growth observed in these pretreated hosts was highly specific, and there was general agreement that the phenomenon had an immunologic basis, but the possibility that these mice had actually been immunized with the killed tissue was not considered until Kaliss demonstrated that specific susceptibility to enhanced tumor growth could be passively transferred by means of serum taken from pretreated mice (*31*). Subsequently it was shown that the activity of the serum was concentrated in the globulin fraction (*32*), and there is now no doubt that this active substance is isoantibody. In our studies we have never observed true immunologic enhancement with tumors susceptible to isoantibody and complement, and it seems both likely and reasonable that resistance to serum antibody is an essential characteristic of all tissues for which enhancement can be demonstrated. In agreement with this concept we have noted on several occasions that antiserums prepared against leukemia tumors will show good neutralization and passive immunity titers when tested against the specific leukoses, but enhancement is observed when these serums are tested against nonleukemia tumors having the same or similar histocompatibility antigens as the leukoses.

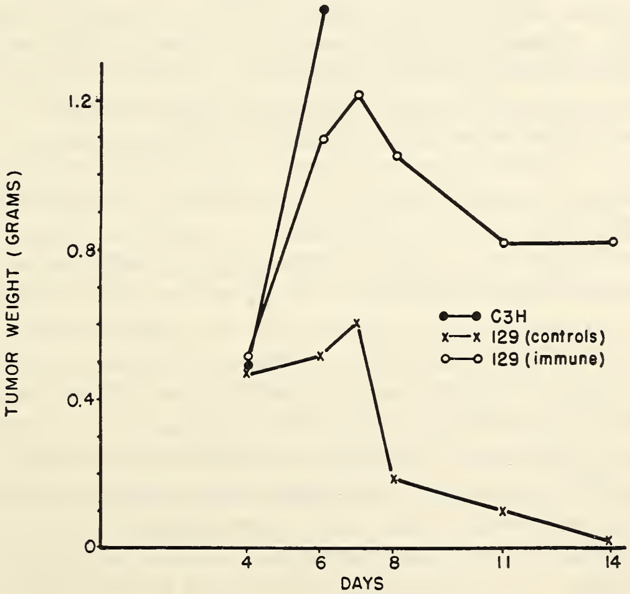
While all the nonleukemia tumors grew better in passively immunized mice than in untreated controls, there were some interesting variations in the effects of the transferred antibody on different tumors. Text-figure 3 shows the differences in the growth of SaI (a strain A fibrosarcoma) in untreated strain A mice, untreated BALB/c mice, and in BALB/c mice that had received intraperitoneal injections of 0.1 ml. of antiserum prepared against SaI. All the mice received 10^6 ascites tumor cells subcutaneously, and, at various time intervals after grafting, 10 mice from each group were killed and the tumors were removed and weighed. In the immunized mice there was an initial inhibition of tumor growth, though it is not clear whether this represents a cytotoxic or cytostatic effect. Subsequently, however, the tumors in these mice grew at a much greater rate than those in the control group. Twenty mice in each of the experimental groups were observed for the ultimate fate of the tumor grafts. All the strain A mice and all the passively immunized BALB/c mice died with tumor, the BALB/c mice surviving an average of 60 days, slightly more than twice the average survival time of the strain A mice. Three of the 20 control BALB/c mice died with tumor at an average time of 72 days after grafting.

Growth of the C3H mammary tumor, H2712, was studied in C3H mice and in normal and passively immunized strain 129 mice. The



Text-figure 3.—Growth of fibrosarcoma SaI in untreated strain A mice, untreated BALB/c mice, and BALB/c mice receiving intraperitoneal injections of BALB/c anti-SaI serum.

results of this experiment are shown in text-figure 4. There was no apparent early inhibition of growth in the immunized mice. The tumor grew much better in this latter group than in the controls, but eventually there were many regressions in the antiserum-treated mice. Actually 14 out of 30 treated mice died with tumor, whereas none of the 30 controls succumbed. The C3H mammary carcinoma, C3HBA, grew much better in antiserum-treated mice than in normal control mice, but



Text-figure 4.—Growth of mammary carcinoma H2712 in untreated C3H mice, untreated strain 129 mice, and strain 129 mice receiving intraperitoneal injections of 129 anti-H2712 serum.

there were no deaths in either group during the course of the experiment. These differences in the occurrence of early tumor inhibition and in the mortality of mice receiving antiserum and tumor are probably due in part to the differences in the amounts of antibody contained in the various antiserums, but as Snell (*5*) has previously pointed out, the rate of growth and homotransplantability of different tumors are probably important in determining enhanceability.

Mitchison (*33*), using SaI, studied the phenomenon of immunologic enhancement, and showed that the level of cellular antibody was lower at 7 days in antiserum-treated mice bearing tumors than in untreated mice grafted with tumor. Kaliss (*34*), working with the same tumor, employed an entirely different and a more sensitive experimental procedure, and found that antiserum-treated mice did, in fact, develop resistance to the tumor graft despite its progressive growth. Kaliss ascribes his observations to some physiologic alterations in the progressively growing tumor, whereas Mitchison has interpreted his results as showing an interference by antibody with the response of the local or draining lymph nodes.

Our results with tumors C3HBA and H2712 support the idea that enhanced tumors provoke an immune cellular response, though it is obvious that this response is either delayed or depressed. The experiments with SaI also indicate that antiserum-treated mice display some resistance to the progressively growing tumors, for the rate of tumor growth is much lower in these mice than in the mice of the indigenous strain, and there is a corresponding difference in the survival times of the two groups. Also, the fact that the survival times of homografts of skin (*35*) or slowly growing tumors are only slightly prolonged in antiserum-treated mice argues against any marked or complete suppression of the normal immune responses of these animals. Moreover, the degree of enhancement that is observed with a given tumor is greatly influenced by the strain and sex of the host mice, and depending on these factors, the mortality among treated mice may vary from 0 to 100 percent. We have often observed that an antiserum prepared in one strain of mice may have only a mild enhancing effect on tumors implanted in other members of that strain, whereas the same antiserum may show striking tumor enhancement in another strain of mice, particularly in a strain more "susceptible" to enhanced growth of the tumor in question. When nonlymphoid tumor cells are mixed with antiserum and injected into compatible mice, there is no apparent increase in the growth rate of the tumor. This is in agreement with the data of text-figures 3 and 4, which show that enhancement means that the tumor in antiserum-treated mice grows faster than the tumor in normal *incompatible* mice, but not as fast as the tumor in genetically compatible mice. In other words, enhancement is not really a stimulation of the tumor to grow beyond its normal rate, but rather a depression of the immune responses of the incompatible host, which permits the tumor to grow in these mice at a rate approaching that observed normally in the indigenous strain. For more detailed discussions on the nature and possible mechanisms of immunologic enhancement, the reviews of Kaliss (*7*) and Snell (*5*) should be consulted.

We may turn now to other reports in the literature in which putative immune serums were found to be without effect on tissues for which they were assumed to be specific. Experiments carried out in tissue culture are difficult, if not impossible, to evaluate, for complement has never been considered in such studies (*36*, *37*). Mitchison (*38*) has reported the results of some passive immunity studies carried out with the 6C3HED lymphoma, and he has concluded from these studies that transplantation immunity is more easily transferred by cells than by antibody in solution. As a matter of fact, Mitchison was not able to demonstrate any transfer at all with serum. These results are in striking contrast with our own data obtained in experiments with the use of the same tumor. There are, however, several objections to the experimental procedures employed by Mitchison. In testing serum, he made the test grafts by trocar into very young (8–10-day-old) mice. A graft of this size would undoubtedly find insufficient complement available in such a small host, even if an optimal amount of antibody was present. It is doubtful that this latter condition obtained, since the serum donors received injections of tumor on only one occasion, and were bled at a 10- to 30-day interval. Unfortunately, no independent test for antibody was carried out. The transfer with lymphoid cells was carried out in adult mice. It is known that these specifically activated cells continue to function and produce soluble or serum antibody in their new hosts, which results in a situation similar to active immunity. As we have already indicated, the concentration of complement is not critical when antibody is continuously synthesized, so that successful transfer of immunity with cells may have been due to the serum antibody which was elaborated by these cells. It is, of course, possible that the immunity was due in part to a cellular mechanism, for there is no reason to suppose that a given cell type is susceptible to humoral antibody but not to cell-bound antibody. However, the data are equivocal on this point and do not provide a conclusive demonstration of passive immunity due to non-serum antibody.

Kidd (*39*) had sometime earlier reported that suspensions of 6C3HED lymphoma cells were neutralized by immune lymphoid cells but not by immune serum. To some extent this constitutes the first clear-cut demonstration of the importance of the draining lymph nodes in the homograft reaction, but, as in Mitchison's experiments, it is impossible to determine whether the activity was due to the direct effect of lymphoid cells on the tumor or to the elaboration of serum antibody in the new hosts.

The studies of Algire and his coworkers using tissues in diffusion chambers, of Billingham *et al.* using skin grafts, and of Mitchison using an A strain sarcoma are far more convincing in their demonstrations of the importance of a non-serum antibody mechanism of homograft destruction. More will be said of these experiments in the succeeding section of this paper, but first I should like to comment on the procedures necessary to establish the resistance of a tissue to the serum antibody-complement system.

The tissue in question should be tested by one or more of the following techniques, with hyperimmune serums prepared, if possible, against the tissue being tested: (1) cytotoxic test *in vitro;* (2) neutralization; (3) passive immunity. If the tissue is unaffected by these tests, it should be established that the serum does contain antibody. When passive immunization results in specific enhancement of homografts, the presence of antibody is evidently established, and our results strongly suggest that resistance of the tissue to serum antibody is also established. Otherwise, the presence of antibody may be determined by hemagglutinin, hemolytic or complement-fixation tests, or by demonstrating neutralization, cytotoxicity, or passive immunity with the serum in question and a tissue known to be susceptible to serum antibody. If antibody can be demonstrated, it should then be shown that it can be specifically absorbed by the tissue under consideration. A tissue that can react with and absorb antibody and is unaffected by such antibody, even when complement is present, is undoubtedly resistant to the direct effects of this immune substance. If passive immunity fails with this antiserum but is successful with specifically activated lymphoid cells, it may be concluded that soluble antibody does not play a primary role in the rejection of homografts of the tissue in question. Transferred cells, however, do produce soluble antibody and it is, at present, impossible to establish experimentally a condition in which cellular antibody is present in the absence of free or soluble antibody. It is, therefore, impossible to rule out some participation of the latter in any homograft reaction. That a graft may be rejected in the absence of demonstrable serum antibody does not affect this conclusion, because (1) failure to demonstrate antibody is often due to the use of insensitive techniques, and (2) a test performed on serum is static in that it records only the level of antibody present at a given moment, that of the time of bleeding. Moreover, serum taken during the time of persistence of the graft or immediately after rejection may not reflect the intensity of the immune response, since a good deal of antibody will be "trapped" by the graft itself. When the lymph nodes or spleens draining a graft are tested, one is dealing no longer with a static system but with a tissue that represents the potential immune response of several subsequent days. The claim that there is a difference in the time of cellular and humoral responses (*11*) is, accordingly, misleading.

I have said nothing about phagocytes in homograft reactions because, insofar as the reaction is specific, little is known of the role of these cells. After the actual destruction of grafted cells, it seems likely that phagocytes play an important part in removing the cellular debris, but, as for participating in the actual cytolytic reaction, their activities are somewhat limited. They are probably important in the removal of homografted red blood cells from the host's circulation, particularly when these cells become sensitized with antibody. Here, also, complement is important, for it is known to enhance opsonization by antibody. For additional information on the role of phagocytosis in transplantation immunity, the review by Gorer (*1*) should be consulted.

THE ROLE OF CELLULAR OR CELL-BOUND ANTIBODY

A number of early investigators were aware of the infiltration of tumor grafts by small round cells, and Murphy (*40*) had become quite convinced that lymphocytes were directly responsible for the rejection of tumor homografts. Unfortunately, most of the early work was carried out with noninbred animals, and the high degree of specificity of tumor-homograft rejection was not appreciated, so that the invading lymphocytes were not looked upon as the products of immune responses, specific in the sense that classical serum antibody was known to be specific. Indeed, the concept of specifically activated cells did not begin to take form until 1942 when Landsteiner and Chase (*41*) reported the successful transfer in guinea pigs of specific dermal sensitivity to simple chemical compounds, by means of washed exudate cells donated by actively sensitized animals. Later, Chase (*42*) demonstrated passive transfer of cutaneous hypersensitivity to tuberculin, and, subsequently, a number of investigators have confirmed these experiments, with a variety of animal species used to study various forms of delayed hypersensitivity. Much of the significance attached to these studies stems from innumerable reported failures to transfer delayed or tuberculin-type hypersensitivity to normal animals by means of serum taken from highly sensitized donors. It has been made clear by many investigators that this failure was not due to the absence of demonstrable serum antibody. Moreover, animals that were passively immunized with serum often displayed anaphylactic sensitivity to the test antigens, despite their lack of delayed cutaneous sensitivity to these same substances. However, the specificity of delayed hypersensitivity, the antigenic nature of the incitants, the necessity for contact with the incitant before acquisition of the sensitive state, the close correspondence in the incubation periods involved in antibody formation and development of hypersensitivity, the long persistence of hypersensitivity and the occurrence of booster or anamnestic responses had for years clearly indicated some sort of antibody mechanism. The successful transfer of this allergic condition by cells of the type known to be involved in immune responses constituted, therefore, an important discovery in modern immunology.

Closely connected with this new line of investigation has been the development of techniques for following the synthesis of antibody by homografted lymphoid cells that had been stimulated with antigen before their removal from the donor animals. Studies of this type were foreshadowed by the investigations of McMaster (*43*), Ehrich and Harris (*44*), and White and Dougherty (*45*), all of whom had earlier demonstrated the importance of lymphoid tissue in antibody production, but the direct stimulus came from the work of Chase, who first clearly demonstrated the elaboration of circulating antibody by transferred or homografted cells of the lymphoid series (*46*). Chase's methods have been modified and used extensively by a number of workers but principally by Chase (*47*), Harris and Harris (*48*), Dixon and coworkers (*49*), and Stavitsky (*50*). These workers have employed cell-transfer techniques in a variety of ingenious experiments aimed largely at the development of a clear understanding of the production of circulating or soluble antibody. Chase was able to demonstrate that the transfer of a given cell suspension could result in both the elaboration of circulating antibody and the development of delayed hypersensitivity. He has, however, set forth a very convincing argument against the assumption of any causal relationship between these two phenomena (*47*). In the study of the immunologic reactions carried out by transferred cells, there are, as Chase (*47*) has previously pointed out, two lines of investigation involved, and the relationship between the two has yet to be uncovered. Recently, though, Pappenheimer has proposed that the specifically activated cell, which mediates delayed allergic reactions, represents an early stage in the production of soluble antibody (*51*). This interesting suggestion is certainly in agreement with many of the reported observations in this field, and it has revived a good deal of interest in a similar proposal made by Dienes and Schoenheit in 1929 (*52*). The hypothesis put forward by Pappenheimer takes into account the considerable number of advances that have been made in the field of immunology since the earlier proposal of Dienes, and Pappenheimer's recent review contains a thorough and provocative discussion of the implications of the proposed relationship.

While the establishment of some metabolic relationship between cell-bound antibody and serum antibody would constitute an important step in our understanding of the over-all immune response, it would not necessarily explain the sharp differences in the biologic activities of these two immune substances. In the fields of both allergy and transplantation, one finds that circulating and cell-bound antibodies of apparently identical specificity can differ profoundly in their reactions with specific antigens. Since both types of antibody appear in the hosts of transferred cells, it is often a matter of great difficulty to separate the effects of the two, and it should be appreciated that any given activity is considered to be a function of cell-bound antibody only after rigorously excluding a primary role of circulating antibody. This is necessitated by the nature of the materials and techniques involved, and it is principally for this reason that we have outlined the scheme for demonstrating the resistance of a tissue to serum antibody.

I should like, now, to consider those cases in which transplantation immunity has been transferred successfully with immune cells but not with immune serum, even when the latter substance was known to contain a relatively large amount of specific antibody. This was first clearly demonstrated by Mitchison, using SaI, a strain A fibrosarcoma (*33*). Immunity to grafts of this tumor could be transferred with lymph-node cells but not with serum containing readily detectable antibody. Subsequently, Kaliss reported that hyperimmune serums actually enhanced the growth of this tumor in foreign strains (*31*), and these two pieces of work provide unequivocal evidence of the failure of serum antibody alone to cause rejection of one type of homograft. Mitchison (*33*) actually worked with several tumors, but the data obtained with most of these are insufficient to rule out the importance of serum antibody. Indeed, we have provided evidence discussed earlier in this paper that at least two of the tumors, 6C3HED and C1498, are readily destroyed by isoimmune serums and complement.

A series of investigations reported by Billingham, Brent, and Medawar (*53*), Billingham and Sparrow (*54*), and Billingham and Brent (*55*) have, in the opinion of most workers, including the present writer, excluded the participation, except in very minor ways, of serum antibody in the rejection of homografts of skin in mice and in rabbits. On the other hand, Billingham, Brent, and Medawar have succeeded in transferring immunity to skin by means of cells.

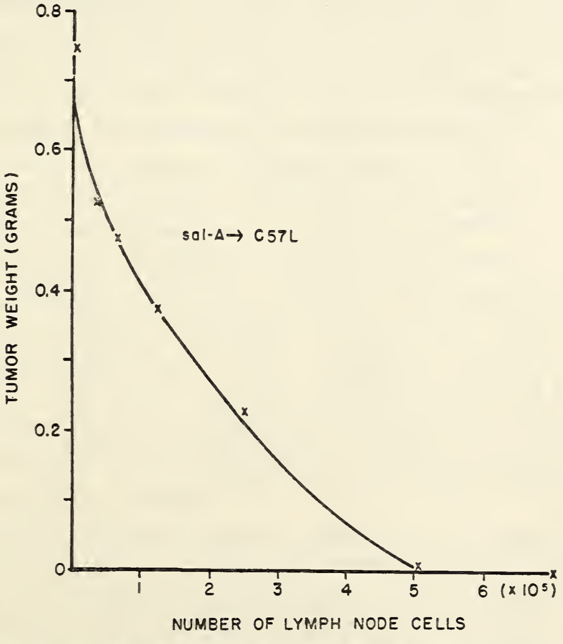
The experiments carried out by Algire and his colleagues, using diffusion chambers, have provided us with interesting and useful data. There are, however, a number of factors that complicate the interpretation of the results obtained with diffusion chambers. The rates of diffusion of isoantibody and complement molecules across the membranes are not known. This is a serious drawback, since, as we have discussed earlier, the rate of cell destruction by antibody and complement is proportional to the concentration of these two substances. With tumor cells it is particularly important to establish that both antibody and complement are as accessible to cells in chambers as to cells in "normal" graft sites. The finding that mammary-carcinoma cells were destroyed, when present in the chamber with splenic cells from immunized animals, does not prove that a soluble antibody was not involved, for, as Mitchison and Dube (*56*) have shown, these splenic cells have the capacity to elaborate circulating antibody. The use of normal tissues in diffusion chambers probably provides data that are less equivocal than those obtained with tumors. Since normal tissue cells generally do not multiply at the high rates characteristic of tumor cells, homografts of the former type would be more vulnerable to low concentrations of complement, provided at least some antibody were present. It seems likely therefore that the resistance of harderian gland and embryonic lung rudiments to serum antibody alone has been established by the diffusion-chamber technique.

Although it is quite firmly established that a variety of tissues are resistant to the cytotoxic effects of isoimmune serums even when high concentrations of complement are present, and that these tissues are, when homografted, destroyed by a cell-bound antibody, there is nothing known of the mechanisms involved in the destruction of homografts by the specifically activated cells. Mitchison (*33*) and Billingham, Brent, and Medawar (*53*) have shown that transferred cells continue to function in their new hosts for at least 30 days, and in some cases the effectiveness of a given number of immune lymphoid cells was found to increase if they were injected 2 or 3 days prior to the application of the test grafts. The studies of Kidd (*39*) and of Algire (*57*) suggest that intimate contact between the immune lymphoid cells and the target cells is essential for graft destruction. Beyond this, we have little experimental data relating to the details of the immune reaction included here.

To a large extent the study of the action of specifically activated cells in destroying homografts has been hampered by the lack of suitable techniques. Too little is known of the reaction to elaborate a technique *in vitro*. The passive transfer of immunity described by Mitchison requires that each host receive the lymph nodes of at least 4 donor animals. This procedure involves a considerable expenditure of time and animals and fails to allow a direct comparison of various tissues from immune animals (it is, in practice, impossible to transfer spleens or total blood from 4 or more donors to 1 host). The diffusion-chamber techniques have, like the passive-immunity studies, provided convincing evidence of the importance of cells in the breakdown of some homografts, but they are, in general, a good deal more time consuming than the techniques used in the study of serum antibody, and while they will undoubtedly find wide application in the study of tissue transplantation, they are not technically suited for a detailed analysis of the role of immune cells in the homograft reaction.

Kidd and Toolan (*58*), in 1950, presented a brief description of a technique in which cells of the 6C3HED lymphoma were mixed with minced nodes of mice that had regressed this tumor, and were injected into C3H mice. Tumor cells mixed with normal lymph nodes grew progressively, killing all the injected hosts. The "sensitized" lymph nodes greatly retarded or prevented the growth of the tumor cells in susceptible hosts. I have already discussed the fact that these experiments do not distinguish between cell-bound and humoral antibodies as the uniquely active immune agents, but because of the relative simplicity of the technique and its potential application to a wide variety of tumor types, we have studied the procedure in great detail, with tumors known to be resistant to serum antibody.

Text-figure 5 shows the results of an experiment with SaI. A group of C57L mice were implanted with this tumor subcutaneously on the right flank. Seven days later the mice were killed and the axillary, brachial, and inguinal nodes were removed from the right side of each mouse. All the nodes were pooled and a suspension of uniformly dispersed cells was prepared in a cytosieve. Serial doubling dilutions of the lymph-node cell suspension were mixed with fresh tumor cells and the mixtures were then implanted subcutaneously in C57L mice, each animal receiving 10^6 tumor cells. Seven days later these mice were killed, and the tumors were removed and weighed. The growth of tumor cells mixed with normal lymph-node cells, in the concentrations used here, was essentially indistinguishable from that of tumor cells mixed with normal mouse serum. There was, on the other hand, a clear relationship between the number of immune lymph-node cells and the inhibition of tumor growth. Complete suppression of growth could be achieved with as few as 5×10^5 lymph-node cells. This represents a donor-to-host ratio of 1:100. Some evidence of immunity could be observed even when this ratio had been reduced to 1:1500. The high sensitivity of this technique and the ease with which quantitative results may be obtained have enabled us to carry out a number of experiments bearing on the nature of the reactions involved. The results of these experiments have not yet been published, but I should like to present here a summary of our findings.



TEXT-FIGURE 5.—Tumor weight at 7 days in C57L mice injected with mixtures of SaI and C57L immune lymph-node cells. Normal C57L lymph nodes had no effect on tumor growth.

Incubation *in vitro* of the mixtures of tumor cells and lymph-node cells for periods up to 6 hours has no effect on the subsequent degree of tumor-cell destruction. This is true even when high concentrations of complement are added to the mixtures. We have been unable to implicate complement in this reaction, and, though we have by no means excluded its participation, it seems certain that the concentration of complement is not nearly as critical in these cell-mediated reactions as it is in neutralization by humoral antibody. When tumor cells and immune lymphoid cells that have been incubated together for 6 hours at 37° C. are treated with isoimmune serum specific for the lymphoid cells, the latter cells are destroyed and the tumor cells, when grafted, grow quite as well as tumor cells that have been incubated with normal lymphoid cells or normal mouse serum. It may be concluded that the immune lymphoid cells have little or no effect on tumor cells *in vitro*, and we have accordingly discontinued the practice of incubation prior to grafting.

The cell mixtures may, of course, be injected into mice of any genetic type, but there are certain host-donor combinations that are of special interest and there are others that can yield only highly ambiguous results. In the former category we may consider, as hosts for the tumor-lymph-node mixtures, mice of (*a*) the strain of origin of the lymph nodes, (*b*) the strain of origin of the tumor, (*c*) the F_1 hybrid obtained in matings of (*a*) with (*b*). Mice of the strain of origin of the nodes are antigenically compatible with the transferred lymphoid cells but are necessarily incompatible with the tumor cells. This latter fact limits the types of tumors that may be tested to those that give good palpable growth in foreign hosts. The use of mice of the strain of origin of the tumor has the disadvantages that the transferred lymphoid cells are themselves destroyed by a homograft reaction, and that the normal tissues of the host can react with the immune cells, which interferes with the tumor destruction. On the other hand, these hosts permit the use of low tumor-cell doses and the testing of slow-growing tumors, which ordinarily show either little or no palpable growth when homografted. Moreover, such hosts are valuable in special types of experiments, as for example in those in which it is desirable to eliminate the possibility of an active immune response to the tumor being tested. The F_1 hybrid mice possess the advantages of being unable to respond actively to either the tumor or lymphoid tissue, and of permitting the testing of virtually all tumors of the genotype of either parent. Unfortunately, the parental lymphoid tissue can and apparently does react against the "foreign" antigens of the hybrid. This is especially noticeable when the lymphoid tissue is taken from immune mice. This does not, of course, prohibit the use of F_1 hybrid mice as hosts, but limits to some extent the range of experiments that can be carried out with them. The choice of hosts for a given experiment is, then, determined in large measure by the type of information desired and the growth rate of the tumor that is used.

Because of the relatively small numbers of immune lymphoid cells that are required to suppress the growth of tumor when this method is used, we have been able to make direct comparisons of the importance of various host tissue in rejecting tumor grafts that have been implanted subcutaneously. Contrary to previously published reports, we have found that the spleen is as important as the draining lymph nodes and that anatomically distant lymph nodes show definite immune activity. Thymus cells, however, have never been found to exert any significant inhibitory effect on the growth of tumor cells.

Using as hosts mice of the strain of origin of the lymphoid cells, we have compared the effects on tumor growth of immune cells administered (*a*) subcutaneously with tumor cells, (*b*) subcutaneously on the side opposite to that in which tumor is implanted, and (*c*) intraperitoneally (the tumor cells being placed subcutaneously). In a series of experiments of this type it was found that immune lymphoid cells placed subcutaneously with the tumor cells were from 5 to 100 times more effective in transferring immunity than when placed intraperitoneally. Immune cells placed subcutaneously on the side opposite to that of the tumor were about 50 percent as effective as cells placed intraperitoneally. These results are consistent with the idea that the immune lymphoid cells must come into direct contact with the tumor cells in order to destroy them. It seems very unlikely that such wide differences in activity would be found, if the active material was a soluble substance elaborated by the cells. We are certainly more inclined to attribute the differences to localization of lymph-node cells at the site of injection. This localization insures a high concentration of active cells at the site of the tumor implant when tumor cells are mixed with lymphoid cells prior to grafting, and at least partially blocks the migration to the tumor of lymphoid cells injected at sites remote from the tumor graft. That immune lymph-node cells are effective in destroying tumor cells, when mixtures are injected into mice of the strain of origin of the tumor, indicates that a considerable number of lymphoid cells must remain at the point of injection, since cells which migrate from this site would be inactivated by the antigens of the host's normal tissues. This fact can be deduced from the observation that even extremely large numbers of immune lymph-node cells are ineffective in conferring immunity on mice of the strain native to the tumor, if the lymphoid cells are injected at a site different from that of tumor injection.

The mechanisms whereby immune lymphoid cells destroy the cells of tissues that are resistant to humoral antibody are entirely unknown. There are some interesting analogies which may be drawn between this type of homograft reaction and the phenomenon of delayed hypersensitivity, but our knowledge of the mechanisms involved in the latter type of reactions is not complete. Moreover, the observation that patients with agammaglobulinemia do not reject skin homografts but do develop tuberculin-type hypersensitivities argues against strict identification of the homograft reactions with those of delayed hypersensitivity.

This leads us to consider briefly the importance of nonspecific tissue damage in graft rejection, for, if a true delayed allergic response were involved, one might expect a considerable amount of tissue to be sloughed as an indirect consequence of an immune reaction. The available experimental data are not conclusive, but the work of the Kleins (*59*), with grafts of mixtures of cells prepared from compatible and incompatible tumors, indicates that individual cells are destroyed by a highly specific mechanism, and Kidd (*39*) has described in detail his observations on the destruction of the cells of a homografted tumor by individual host lymphocytes. Both sets of data must, however, be considered carefully since they deal exclusively with the mouse, an animal notorious for its resistance to the development of delayed hypersensitivity. Experiments carried out with guinea pigs would probably be more instructive, though Wallace has recently demonstrated that the cells of rats, known to be highly refractory to the establishment of delayed hypersensitive states, can transfer delayed reactivity to guinea pigs (*60*). Rats, and very likely mice, have, then, the capacity to elaborate the immune substances that mediate delayed hypersensitive responses, but evidently are constitutionally incapable of displaying typical responses. It may be concluded that some types of homograft rejection involve the direct participation of immunologically activated cells similar to or identical with those involved in delayed allergic reactions. Homograft reactions are evidently more complicated than the allergic responses in requiring some additional immune factor. At present it seems best to refrain from speaking of transplantation immunity as a form of hypersensitivity. To equate the two phenomena may be like considering immunity to pneumococcal pneumonia as a form of anaphylactic sensitivity. Both these activities are mediated by serum antibody, but the mechanism of opsonization is in many ways distinct from the reactions culminating in the release of the pharmacologically active compounds that produce anaphylactic symptoms. Similarly, an immunologically activated cell may provoke an allergic inflammatory response when it reacts with its specific antigen, but this does not mean that the same cell cannot also mediate an entirely different reaction. Indeed, there is no reason to assume that both reactions cannot be carried out simultaneously. The regression of homografts of those ascites tumors that are resistant to serum antibody is an instance in which homograft reactions proceed in the absence of allergic symptoms. In other cases an allergic component may be prominent and may even lead to some nonspecific destruction of the tissues of the host as well as that of the graft. However, when cortisone is used to suppress the inflammatory symptoms of the reactions in an immune animal, the actual destruction of grafts is only slightly affected. In other words, a specifically activated lymphoid cell may combine with and aid directly in the destruction of a grafted cell; coincidentally, the combination may incite an inflammatory response, the intensity and importance of which may vary with the type of homograft and with the species and physiologic status of the host.

The idea of a direct reaction between graft cells and lymphoid cells may be objected to on the grounds that passive transfer of cellular immunity is more effective when the lymphoid cells are administered several days prior to grafting and also on the grounds that reactions *in vitro* between active lymphoid cells and their target cells are difficult, and perhaps impossible, to demonstrate. However, most work of this type has been done with cells taken from lymph nodes that have very recently drained the site of a foreign graft. It seems likely that many of these cells are immature in the sense that they have not, at the time of excision, reached a state of maximum immunologic competence. It might be expected that, on reaching the latter state, the cells would move out from the node. Also, the failure of these cells to interfere with specific tissues *in vitro*, as for example in tissue culture, probably reflects the requirement for cofactors not supplied in the test system. Such cofactors might be represented by an immune substance (humoral antibody) or some substance such as complement. Lastly, we do not know what percent of the cells in a given lymphoid organ are actually specifically activated, but if it is small, it is to be expected that a good deal of difficulty will be encountered in observing any reactions *in vitro*.

The analogies that may be drawn between delayed hypersensitivity and homograft reactions are helpful in understanding the factors determining whether a homograft response will consist primarily of the elaboration of humoral or cellular antibody. However, it is not the purpose of this discussion to consider the nature and significance of the antigenic stimulus involved in the homograft problem, and the interested reader is referred to the reviews mentioned earlier. I should like merely to point out that the conception of cell-bound antibody has now become important in considerations of delayed hypersensitivity, homograft reactions, autoallergic diseases, and the immunity of agammaglobulinemic patients to certain infectious diseases, particularly those of viral origin. The study of the induction and modes of action of specifically activated lymphoid cells is clearly one of the most important problems in immunology.

In discussing this very important and complex problem of the immune mechanisms involved in graft rejection, I have, for convenience, summarized and rephrased a number of ideas and hypotheses taken from diverse immunologic studies. In so doing I have found it impossible to acknowledge in detail all the sources of these concepts, but the bibliography that follows contains, for the most part, all the sources from which I have drawn.

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Session III. Biology of Cancer

Chairman: Harry S. N. Greene, *Yale University School of Medicine*

Speakers:

Harry S. N. Greene. *A Virus Etiology of Cancer?*

Jacob Furth, Untae Kim, and Kelly H. Clifton. *On Evolution of the Neoplastic State; Progression from Dependence to Autonomy.*

Austin M. Brues. *Prospects for a Systemic Therapy of Cancer.*

A Virus Etiology of Cancer?[1]

HARRY S. N. GREENE, *M.D., Department of Pathology, Yale University School of Medicine, New Haven, Connecticut*

FOR a number of years a large portion of the work in my laboratory has been centered on a discrepancy observed in the biological behavior of ordinary tumors and tumors associated with a virus etiology. The discrepancy concerns transplantation reactions and constitutes a good jumping-off-place for a discussion of the topic assigned me by Doctor Kaliss: "A Virus Etiology of Cancer?"

Many years ago it was observed that the transplantability of spontaneous rabbit tumors was directly related to their duration in the primary host. Early tumors could not be transferred to normal animals, whereas tumors obtained after metastasis had occurred were readily transplantable. The subsequent occurrence of a large number of spontaneous tumors in the colony made a detailed investigation of this relationship possible. Biopsy specimens obtained at monthly intervals throughout the course of disease were subjected to various types of transplantation. In general, 4 types of transplantation were attempted: (1) autologous, or transfer back elsewhere in the same individual; (2) homologous, or transfer to normal, unrelated individuals of the same species; (3) transfer to other tumor-bearing animals; and (4) heterologous, or transfer to normal animals of a different species.

The results are shown in table 1. Briefly, autologous transfer was always successful, even with the earliest tumors. In sharp distinction, homologous transfer was successful only late in the course of the tumor and was followed rapidly by death from metastasis. The results of heterologous transfer paralleled those of homologous transfer and all tumors that grew in normal unrelated rabbits also grew when transplanted to guinea pigs.

TABLE 1.—Transplantation reactions during developmental course of rabbit cancers

			Tumor-bearing animals		
				Homologous	
Primary tumor	Homologous	Heterologous	Autologous	Identical type	Different type
Before metastasis	—	—	+	+	—
After metastasis	+	+	+	+	+

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[1] Presented at a symposium on normal and abnormal differentiation and development, held in celebration of the 30th anniversary of the Roscoe B. Jackson Memorial Laboratory, June 15–17, 1959, at Bar Harbor, Maine.

The transfer of early tumors to rabbits bearing spontaneous tumors of the same type paralleled the results of autologous transfer, and takes invariably occurred. On the other hand, the results of transfer to rabbits bearing spontaneous tumors of dissimilar types were not different from those of homologous transfer to normal animals, and no takes occurred.

It was inferred from these experiments that the ability to survive in the environment of a normal animal is a developmental acquisition and not a property of the tumor from its inception. Prior to the attainment of this property, however, the tumor does possess the ability to survive in the primary host or in other animals bearing similar tumors. Thus, at this stage, survival is dependent on the special constitutional status of the spontaneous tumor-bearing animal. The factors operative in this status are not operative in normal animals and, accordingly, the tumors cannot be transferred to normal animals. Later in the course of development, marked by the simultaneous attainment of the ability to metastasize, the tumor becomes independent of these factors, or autonomous, and will survive in their absence in normal animals.

A similar situation with respect to dependency and autonomy characterizes the development of tumors in mice and man as well as in the rabbit. Dr. Jacob Furth has worked extensively with the evolution of mouse tumors, particularly with those of the endocrine organs (*1*). It should be emphasized, however, that phases of dependency and autonomy are not limited to endocrinological tumors, but also distinguish the development of other mouse tumors.

A typical example illustrating transplantation reactions during the development of a spontaneous tumor in a C3H mouse is shown in table 2. Transfer of early biopsy specimens to unrelated mice and other alien species gave no takes, but after the occurrence of metastasis, both homologous and heterologous transplantations were successful. Furthermore, identical results were obtained with methylcholanthrene-induced tumors.

An investigation of human tumors from this point of view has necessarily been limited to heterologous studies. Furthermore, the presence or absence of metastasis at the time of transfer can only be determined by the duration of life after operation. Table 3 summarizes the results of transfer of 123 human tumors in relation to survival. All the patients with heterotransplantable tumors are now dead, whereas 79 percent of the individuals with nonheterotransplantable tumors are still living (more than 5 years after transfer).

Table 2.—Transplantation reactions during the course of a spontaneous tumor in a C3H mouse

	C3H		DBA		C57BL		A		Bagg		Guinea pig	
	Number	Takes	Number	Takes	Number	Takes	Number	Takes	Number	Takes	Number	Takes
1st Biopsy	5	4	5	0	5	0	5	0	5	0	10	0
2d Biopsy	5	5	5	0	5	0	5	0	5	0	10	0
Autopsy	5	5	5	5	5	3	5	4	5	4	10	8

Table 3.—Results of heterologous transfer of human tumors in relation to survival

	Tumors	
Patients	Heterotransplantable	Nonheterotransplantable
Dead	65	12
Living	0	46

It has been concluded from these experiments that, with reference to biological properties, cancers of rabbits, mice, and man are not sudden transformations of normal cells but represent the final step in a developmental process. During the greater part of their course, the tumors are dependent in nature, their continued existence being conditioned by factors peculiar to the tumor-bearing individuals. Autonomy, or the ability to survive in the absence of such factors, is a late development and coincides with the attainment of the ability to metastasize.

With few exceptions, the nature of the factors on which the development of a tumor depends remains obscure. Experimentation with uterine and mammary tumors in rabbits suggests that, in these special cases, the factors are endocrinological. Rabbits bearing such tumors show widespread endocrine changes suggestive of the continued action of estrogenic hormone, and dependent tumors of both varieties will survive transfer to estrogenized animals. It should be emphasized, however, that these are special cases and that estrogenic hormone does not supply the factor on which the growth of tumors in other tissues depends. Doctor Furth has conclusively shown the dependency of many developing endocrine tumors in mice on hormonal secretions, and these are also highly specific. In man, it seems clear that developing tumors of the prostate gland are dependent on an androgenic hormone, but this hormone does not operate as a conditioning factor in the development of other tumors. Accordingly, it would appear that different tumors depend on different conditioning factors.

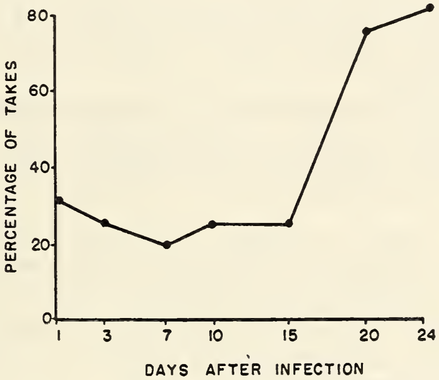
It has also been shown in rabbits that the factors concerned in tumor development are constitutional in distribution and not localized at the site of the primary growth. Fragments from early dependent tumors have been transferred to various parts of the body, and serial biopsies with transplantation studies have been carried out at intervals throughout the remainder of the rabbit's life. Autonomy was attained at all sites of autologous transfer, and no significant variations in the time of attainment distinguished the primary site from other regions.

In brief, the course of all the various tumors studied in rabbits, mice, and man have consisted of two phases: a phase of dependency and a phase of autonomy. The factors concerned in dependency were specific and constitutional in nature.

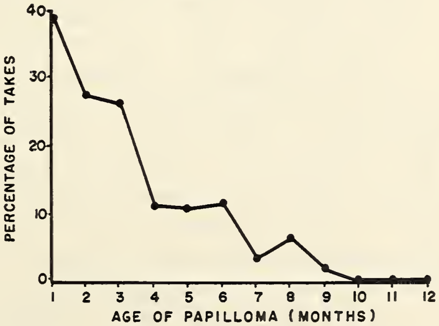
The point to be emphasized in the present context is that the situation with respect to the best-known virus tumors, the Shope papilloma and the Rous sarcoma, is quite different. Both these tumors possess the ability to survive and grow on homologous and heterologous transfer from their earliest stages of development. Transplantability is present from inception, yet metastasizability is acquired only after an extended developmental course.

Not only are the two tumors transplantable from their first clinical appearance, but adult tissues infected *in vivo* with the respective viruses can be removed within 3 minutes of inoculation and successfully grown in both homologous and heterologous hosts. In some instances, particularly in the adult rabbit skin infected with the Shope virus, the tissue persists unchanged for many months, but usually within 10 days the typical morphological changes associated with the disease become apparent in the transplant.

The heterologous transplantability of the Shope lesion throughout its course has been determined and is plotted in text-figures 1 and 2. Text-figure 1 shows the incidence of takes with papilloma induction of infected adult rabbit skin obtained by biopsy at daily intervals and transplanted subcutaneously to DBA mice and to hamsters. Distinct papillary formations were usually apparent in the skin of the infected rabbits by the 24th day, and, thereafter, biopsies and transplantation experiments were carried out at monthly intervals. The results of the latter series of transfers are shown in text-figure 2.



TEXT-FIGURE 1.—Incidence of takes with papilloma formation after heterologous transfer of adult rabbit skin infected *in vivo* with the Shope virus.



TEXT-FIGURE 2.—Incidence of takes after heterologous transfer of papilloma tissue.

The points to be emphasized are that adult rabbit skin becomes heterotransplantable immediately after infection with the Shope virus and, in most instances, is converted to the typical papilloma in the alien host. The papilloma itself is also heterotransplantable from inception, but the incidence of takes declines steadily throughout its course until the 10th month when transfer is no longer successful. It is pertinent to subsequent discussion that the loss of transplantability is coincident with the appearance of morphological tissue changes characteristic of cancer. The lesion remains nontransplantable, despite such histological changes, for the following 3 to 4 months and then suddenly regains transplantability with the attainment of the ability to metastasize.

It has been noted by Syverton *et al.* that the recovery of virus from the papillomas of wild cottontail rabbits depends on the developmental stage of the tumor. They found that 12 of 40 papillomas examined yielded virus, but that no virus could be obtained from 106 that were histologically diagnosed as carcinoma. The possibility arose from this and other evidence that transplantability might be determined by the presence of virus, and, accordingly, experiments were carried out in which papilloma tissue, obtained at age periods of low transplantability, and nontransplantable "cancer" tissue were reinfected with wild virus before transplantation.

It is highly significant that after such treatment, papilloma tissue of low transplantability became readily transplantable and that nontransplantable tissue in the stage of histological cancer regained transplantability.

Furthermore, in several instances, papilloma tissue from cottontail rabbits tested and found to be noninfectious and presumably virus-free was transplanted subcutaneously to hamsters both without treatment and after reinfection *in vitro* with wild virus. No takes were obtained from transfer of the untreated tissue while the reinfected papilloma grew in the majority of recipients.

Although the behavior of the Rous sarcoma in the chicken prevents comparable serial transplantation experiments, investigation suggests a similar situation. Adult chicken muscle is transplantable in the brains of mice and rabbits immediately after infection and in these sites is rapidly converted to the Rous sarcoma. The sarcomatous lesion induced in the adult chicken is also transplantable from inception and gives rise to a high percentage of takes. The high incidence of takes continues until the chicken dies, and never has reversion to nontransplantability been noted. It should be emphasized, however, that the interval studied rarely exceeds 2 months and throughout the period the growth remains infectious and contains virus in high titer. It is significant to the point under discussion, however, that the low-virus tumors of Bryan and the nonfilterable tumors occasionally found in older chickens will not survive transfer to alien species.

It is suggested from such evidence that the intracellular virus is analogous in its operation to the constitutional factors operative in the dependence of other tumors, and that the transplantability of the Shope papilloma and the Rous sarcoma in normal animals is conditioned on the concomitant transfer of their dependent factors.

A conception of the virus as a conditioning factor, analogous in its action to the constitutional factors concerned in the dependence of other tumors and located in the cell rather than in the host, implies that transfer of an infected cell would necessarily be accompanied by transfer of the factor on which its growth depends. Thus, the special environmental conditions requisite for the growth of other tumors transplanted during dependent phases of development are replaced by intracellular components in virus tumors and, in this respect, the survival and growth of transplants are independent of the constitutional status of the host. The final epidermoid carcinoma-metastasizable stage of the Shope papilloma is characterized by the absence of detectable virus, and the ability of the tumor to survive heterologous transfer at this period of development is evidence of the attainment of autonomy or independence of conditioning factors, such as distinguishes the metastasizable phases of other nonvirus tumors.

One of the many problems arising from such considerations concerns the mechanism of disappearance of the Shope virus during the papilloma-carcinoma sequence, and one experiment in particular is pertinent. In brief, this consisted of the infection *in vitro* of normal adult rabbit skin, papilloma tissue, and epidermoid carcinoma tissue with cottontail virus; transplantation of the infected material to the brains of rabbits immune to the virus; and a test of the resulting growths for the presence of infectious virus.

Despite residence in the immune environment for a month, both the papilloma derived from the skin grafts and the transplanted papilloma tissue contained infectious virus. In sharp contrast, infectious virus could not be demonstrated in transplants of the epidermoid carcinoma, though a high titer was present in control transplants to normal, nonimmune animals. Inasmuch as the virus is intracellular, the implication is that immune bodies failed to gain entrance into the cells of either the adult skin or the papillomas, but did gain entrance into cells of the carcinoma.

Additional experiments were designed to test the suggestion that the development of autonomy is associated with an increased ability of the cell to take up protein from the plasma. Radioactive glycine was incorporated into serum protein and injected into rats bearing the Walker carcinosarcoma 256, and the uptake by the tumor was found to be 2½ to 10 times that of other tissues. Again, after the injection of antibody against bacteriophage into hamsters bearing a malignant melanoma, extracts of the tumor and of various organs were plated with phage and bacteria. The high colony counts in plates containing tumor extracts, in contrast to the low counts in plates containing extracts of other tissues, demonstrated their differential antibody content.

Accordingly, an increased permeability of the cancer cell to plasma proteins is suggested as a possible mechanism to account for the absence of virus from the epidermoid-carcinoma stage of the Shope papilloma.

If this suggestion is correct and applicable to other cancers, the failure to demonstrate virus in such material cannot be taken as evidence against a virus etiology. The cells of the developing neoplasm become permeable to antibody with the attainment of autonomy, and any virus present is destroyed.

In view of this possibility and the failure to demonstrate virus in the vast majority of animal and human cancers tested, the search has been extended to the dependent phase or so-called precancerous lesion. Transplants of embryonic tissues rather than the tissues of adult animals have been used as hosts, for it has been found that the susceptibility of such tissues, particularly with reference to the Shope virus, is much greater than that of their adult counterparts.

For example, embryonic rabbit skin can be infected *in vitro* with Shope virus in a much lower titer than is required for the infection of adult skin, and when such skin is transplanted to adult rabbits, hamsters, mice, or guinea pigs, the characteristic papilloma develops. Moreover, embryonic rat skin treated in the same manner and transplanted to adult rats also reacts with typical papilloma formation. In the latter case, the adult tissues of the species are completely resistant to the virus, yet their embryonic counterparts are susceptible.

A large number of human tumors in their dependent phases has been tested for the presence of virus. That is, embryonic counterparts of the affected human tissue derived from a number of animal species have been treated *in vitro* with tumor filtrate, transplanted to adult animals of the respective species, and subsequently examined for histological evidence of neoplastic change. In no instance has such evidence been found and, although negative results are of little significance in such studies, it is suggestive that two known virus lesions from man, molluscum contagiosum and the Strauss-Bunting wart, have been induced in embryonic guinea-pig skin by the technique used.

On the basis of the observations described, it has been concluded that the Shope papilloma and Rous sarcoma are virus-dependent tumors. Present results suggest that these are unique tumors and, despite continued search, their counterparts have not been found among the many neoplasms studied in rabbits, mice, and man.

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On Evolution of the Neoplastic State; Progression From Dependence to Autonomy [1, 2]

JACOB FURTH,[3] UNTAE KIM, *and* KELLY H. CLIFTON,
Children's Cancer Research Foundation, Children's Medical Center, and the Department of Pathology, Harvard Medical School, Boston, Massachusetts

THERE may be many definitions of cancer, but to us there is only one that fits all types: Cancer is unrestrained proliferation of cells that are normally limited.

Numerous cell types live side by side in the body, capable of perpetuating themselves within limits, the number of each being maintained by a precise regulatory mechanism, homeostasis. The cancer problem on its basic level is one of homeostasis, with the various cell types linked to the dynamics of cell replication, mode of development, and the character of inheritable modifications of cells. It is now an old story that homeostatic disturbances can result in excessive proliferation of a cell type, which, if localized, can yield a progressively growing tumor. Further progression of such tumors is at first conditioned on maintenance of the homeostatic disturbance until autonomy is acquired.

Since homeostatic forces are best known in the domain of endocrines, we shall limit our discussion to examples of endocrine disturbances that can lead to cancer.

The examples surveyed in table 1 are now fairly well known. We shall review only a few informative examples and cite some new, relevant observations as a basis for discussion. Our thoughts will be directed to the type and character of neoplasms resulting from disturbances of homeostasis of cell numbers, to the fundamental differences between normal, autonomous, and dependent cells, and to the nature of the inheritable alterations of cells. Briefly the questions are: How do cancer cells originate and what is their character?

Mammary tumors and mammotropes.—Three recent findings related to mammary cancers deserve discussion: (1) the induction of hormone-responsive mamary tumors in the rat by ionizing irradiation (*22*); (2) the ease of induction of similarly hormone-responsive mammary tumors by 3-methylcholanthrene (MCA) (*23*, *24*); and (3) recognition of the primacy of the pituitary cell, called mammosomatotrope or mammotrope, in stimulation of the mammary gland [4] (*25*).

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[1] Presented at a symposium on normal and abnormal differentiation and development, held in celebration of the 30th anniversary of the Roscoe B. Jackson Memorial Laboratory, June 15–17, 1959, at Bar Harbor, Maine.

[2] Supported by grants from the National Cancer Institute, National Institutes of Health, Public Health Service, and the U.S. Atomic Energy Commission.

[3] *Present address:* Roswell Park Memorial Institute, Buffalo, N.Y.

[4] Kim, U., Clifton, K. H., and Furth, J.: To be published.

TABLE 1.—Induction of dependent tumors and their autonomous variants

Organ and cell type	Species	Homeostatic imbalance: Hormone	Homeostatic imbalance: Procedure	Dependent tumors: Induction period (*month*)	Dependent tumors: Latency of graft (*month*)	Autonomous tumors: Passage of origin	Autonomous tumors: Latency of graft* (*month*)	References
Pituitary								
Thyrotropes (Tt)	Mouse	Thyroid lack	Thyroidectomy	10–18	4–9	2d–on	2–11	*1, 2*
Mammotropes (Mt)	Rat	Estrogen+	Pellet	6–14	5–11	2d or 3d	1. 5–4	*3*
Ovary								
Most cells	Mouse	Gonadotropic+	Portal graft	6	<2–4	2d or 3d	1	*4*
	Mouse	Gonadotropic+	Portal graft			Primary host	<1–4	*5*
Testis								
Leydig's cells	Mouse	Estrogen+	Pellet	8–10	<6–8	Primary–3d	<1–4	*6, 7, 8*
Thyroid	Rat	Thyrotropic+	Low I diet	6–12	—	—	—	*9*
Epithelium	Rat	Thyrotropic+	Thiouracil	23	<5	4th–6th	0. 2	*10*
	Mouse	Thyrotropic+	Thiouracil	18	<4–9	3d–9th	—	*11*
	Mouse	Thyrotropic+	TtT graft	10	2–10	None yet	—	(†)
Mammary gland								
Epithelium	Rat	Estrogen+	Pellet	7–11	—	—	—	*12*
	Mouse	Of pregnancy	Pregnancy	9–27	<1	Primary, 1st	<1	*13, 14*
Kidney								
Epithelium	Hamster	Estrogen+	Pellet, injection	9–12	7–12	14th		*15, 16*
Adrenal								
Cortical cells	Mouse	Gonadotropic?	Gonadectomy	6	—	Primary, 2d	1–4	*17, 18*
Uterus								
Smooth muscle	Hamster	Estrogen and androgen+	Pellet	7–13	?	—	—	*15, 19*
Epididymis								
Smooth muscle	Hamster	Estrogen and androgen+	Pellet	11–20	?	—	—	*15, 20*
Uterine and abdominal connective tissue	Guinea pig	Estrogen+	Injection, pellet	2–4	—	—	—	*21*

*First few passages. Latency is progressively shortened with further grafting.

†Ghera, N. H., Pullar, P., and Furth, J.: Unpublished data.

Induction of mammary tumors by irradiation.—Whole-body ionizing irradiation results in the development of mammary tumors in both male and female rats.

Table 2 is a summary of some of the work of Shellabarger *et al.* (*22*) and personal communication. In unirradiated rats mammary adenomas are very common, but carcinomas are rare and neither develops commonly before the age of 1 year. The table shows that, after 400 r whole-body irradiation, the majority of female animals develop mammary tumors within 11 months. Tumor induction is influenced by estrogenic hormones. Frequency in females is much reduced and in males increased by gonadectomy. Implantation of ovaries in oophorectomized animals counteracts the inhibitory effect of gonadectomy.

TABLE 2.—Induction of mammary tumors in rats by X rays

Treatment	Females	Males	
	At 11 months (*percent*)	At 11 months (*percent*)	At 16 months (*percent*)
None	2	0	0
400 r	79	18	54
Gonadectomy + 400 r	23	38	62
Gonadectomy + 400 r + ovarian implant	82		
Head shielded, 400 r	81		

These observations recall the early phase of research on the influence of estrogens on mammary-tumor development in mice. When oophorectomy was performed early in life so as to minimize or eliminate estrogenic stimulation, mammary-tumor development was greatly retarded. Mammary-tumor incidence was decreased somewhat when oophorectomy was performed in young adults (*26*). These findings will probably apply to irradiation-induced mammary tumors in rats; *i.e.*, complete exclusion of sources of estrogen early in life may prevent mammary-tumor induction by irradiation.

The high percentage of mammary tumors in castrated male rats suggests that elimination of inhibitory androgens, by castration, may result in enhancement of estrogen production by the adrenals. It is our supposition (the experiment has yet to be performed) that combined adrenalectomy and gonadectomy in males will also prevent mammary-tumor induction by irradiation. Ionizing radiation initiates carcinogenesis by causing an inheritable change in cells. Secondary promoters, as estrogens or mammotropins (mammotropic hormone), are required for actuation of the neoplasm.

The tumors induced by irradiation are of the types that occur in untreated rats at a later age and at a much lower frequency. The tumors occurring most commonly in untreated rats are fibroadenomas. In contrast, about one third of those induced by ionizing irradiation are carcinomas, and a smaller number are sarcomas, the latter exceedingly rare in untreated rats.

Although the total number of animals is small, the data of Shellabarger *et al.* [(*22*) and personal communication] suggest a linearity of response in the dose range of 25 to 400 r. The authors interpret these data as supporting the somatic-mutation theory of cancer by irradiation. However, studies of mammary-tumor induction by X irradiation with values below 25 r are wanting. It has yet to be proven that the body fails to compensate for irradiation damage by small doses. Although the heritable somatic modification involved in irradiation tumorigenesis of the mammary gland is best explained as a modification of deoxyribonucleic acid (DNA), it is also conceivable without it. Aside from this, the work of Shellabarger *et al.* shows that the development of mammary tumors is markedly influenced, perhaps even conditioned, by estrogenic stimuli and could probably be counteracted by androgens after the presumed somatic mutation had been induced.

Mammary-tumor induction in rats by 3-methylcholanthrene.—Huggins *et al.* (*24*), extending the observations of Shay *et al.* (*23*), have shown that it is possible to produce mammary tumors in almost all rats by feeding them MCA. The data of Huggins *et al.* indicate that most of these tumors are hormone-responsive. They are stimulated by estrogens and inhibited by androgens.

Kim, Clifton, and Furth confirmed and extended these observations (to be published). Table 3 summarizes 5 experiments, 1 with castrated males and 4 with intact females. Mammary tumors were induced in 54 to 100 percent of the females. No mammary tumors were induced in castrated males by similar treatment. Tumors were more frequent in the group in which only 18 treatments were given. When treatment was prolonged, some animals failed to develop tumors.

TABLE 3.—Mammary-carcinoma induction by MCA feeding in rats

Number	Strain*	Sex	Number of treatments (*twice weekly*)	Percent at 3 months post treatment	Mean latency (*days*)
17	F	♀	14	54	131
23	F	♀	14	95	115
27	F	♀	13	85	116
18	W	♀	9	100	98
20	F	⚦†	14	0	—

*F = Fisher rats; W = Wistar rats.
†⚦ = Castrated males.

A biphasic effect of estrogens and carcinogenic hydrocarbons is evident from studies of several investigators. In large doses a carcinogen can be carcinolytic, as shown experimentally by Haddow (*27*), by Haddow and Robinson (*28*), and, in patients, by Huggins and McCarthy (*29*). This applies to estrogen, normally a physiologic stimulant, when used in very large quantities (*30*).

Tumors induced by MCA are malignant, whereas those occurring spontaneously are overwhelmingly benign, and those induced by radiation are predominantly benign. Nevertheless, the majority of MCA-induced tumors are hormone-responsive, though not yet proven to be hormone-curable.

The study of Huggins *et al.* (*24*) was concerned with primary carcinomas. Kim *et al.* compared the behavior of primary tumors with first-generation grafts in highly inbred isologous rats. The analytic possibilities of a primary tumor are much limited. The data, surveyed in table 4, show an excellent parallelism in behavior of primary tumors and first-generation grafts. All tumors proved nearly 100 percent transplantable. Of seven strains listed in this table all but one were hormone-responsive.

TABLE 4.—Comparison in responsiveness of primary *versus* grafted mammary tumors

		Treatment		
Tumor*		Oophorectomy	Hypophysectomy	Mammotropic‡ hormone
HW–4	Primary	†	++	
	Grafted	++	+	
RW–1	Primary	++		
	Grafted	++	+	
HF–5, 9 & 10	Primary		+++	++
	Grafted		+++	+
HF–2	Primary		++	
	Grafted	+	+	
HF–7	Primary		0	
	Grafted	0	0	

*H = MCA-induced; R = radiation-induced; F = Fisher rats; W = Wistar rats.

† + = Degree of inhibition (+ = moderate retardation of growth; ++ = arrest of growth; +++ = regression). 0 = no response.

‡In mammotropic hormone column: + = slight stimulation; ++ = moderate stimulation.

The significance of mammotropes on the stimulation of mammary glands.—Another part of the study of Kim *et al.* was to test the supposition that hypophysectomy is beneficial because it removes the pituitary mammotrope which mediates the action of estrogens (*25*). Hypophysectomy caused regression of both primary and grafted tumors of several strains. However, when autonomous but highly functional pituitary mammotropic tumors were grafted on these rats, growth of the mammary tumor was resumed.

More detail about the effect of various types of hormonal treatment on carcinogen-induced mammary tumors is shown in table 5. Of over 20 transplantable tumor strains established, only 5 of 15 failed to respond to oophorectomy, and 5 of 16 to hypophysectomy. Grafted mammotropic tumors aggravated all 5 strains by the mammotropic hormone(s) they secreted.

In males the 9 tumor strains tested grew very poorly. They remained localized at the site of the graft, forming tiny, firm nodules composed of stromal fibroblasts with pyknotic epithelium, while in female siblings

TABLE 5.—Behavior of 16 carcinogen-induced mammary-tumor strains

Treatment	Number tested	Inhibition*				Stimulation
		0	+	++	+++	+++
Hypophysectomy	16	5	3	5	1	
Oophorectomy	15	5	4	4	2	
Adrenalectomy	8	7				
Androgen (♂)	12	3	4	5		
Mammotropic hormone	5					5

*0 = No response; + = moderate retardation of growth; ++ = arrest of growth; +++ = regression.

or in mammotropic tumor-bearing males they grew progressively, forming bulky tumors (figs. 1, 2, and 3). The mechanism of presumed androgen inhibition remains to be studied.

Earlier experiments indicated that estrogens do not have mammary-gland stimulating activity in the absence of mammotrope (*25*). Figure 4 shows the atrophic mammary gland of an estrogen-treated, hypophysectomized animal, and figure 5 that of a hypophysectomized animal treated with mammotrope in addition to estrogen. Estrogen can stimulate the mammary gland only in the presence of mammotrope, but the mammotrope can do so in the absence of estrogen.

In the mammary gland stimulated by mammotropin over long periods of time, tumor nodules arise, as shown in figure 6, but none of these have proven autonomous. Persistent efforts to drive these nodules to autonomy, by repeated resection of mammotropic tumors to maintain sustained mammary-gland stimulation, have been unsuccessful. Thus, we conclude that mammotropin alone is not capable of inducing mammary tumors in rats.

Mammary-tumor induction in rats as compared to that in mice.—All three lines of recent research indicate the superiority of the rat in the study of mammary tumors. Mammary tumors of mice are almost invariably autonomous—nonresponsive; those of rats mostly hormone-responsive. Is this due to association of virus with the mouse tumors and its absence in the rat?

Such a daring supposition calls for verification. Electronmicrographic studies of Bernhard (personal communication) suggest that even the so-called virus-free strains of mice carry the mammary-tumor agent in masked form. Mühlbock and Boot (*31*), however, believe that the milk factor acts only as a nonessential accelerator in the genesis of mammary tumors, the main factors being ovarian and hypophyseal hormones and genetic susceptibility. On the other hand, proof of the absence of the mammary tumor agent in rats and in man is required, and this is next to impossible to furnish.

Be this as it may, about one half of human tumors at the terminal phase and most rat carcinomas, although autonomous, are somewhat hormone-responsive. It is probable that, when tested early in the disease, most human tumors will be found to be hormone-responsive

and that responsiveness will be greater than in those analyzed at the terminal stages.

Even if the induction of tumors by MCA in the rat is virus-mediated, it does not necessarily follow that these tumors are virus-induced. In rats spontaneous carcinomas are rare but, as Huggins *et al.* (*24*) have shown, they can be readily induced by MCA. The induced tumors vary in character among themselves, but are not contagious and die with the animal. They might yield a cell-free filterable transmitting agent, but would it not be more proper to regard MCA as their causative agent rather than the filterable principle?

Forces maintaining mammary tumors.—In the genesis of mammary tumors, two forces can be clearly recognized: (1) an initiating force which, in rat tumors, may be a mutagen, as X rays or MCA, and (2) stimulating and restraining forces as are the hormones. The carcinogen-induced tumor cell is autonomous but usually still responds to its physiologic regulators.

Thus, there are two possibilities for control of mammary tumors: (1) elimination or reduction of the carcinogen, and (2) alteration of the homeostatic forces. Examples of this, now in clinical use, include removal of the source of stimulating hormones; *i.e.*, gonadectomy, hypophysectomy, and adrenalectomy. Another approach would be the utilization of specific affinities of hormones for certain tumor cells by attaching to them nonspecific, carcinostatic or carcinolytic agents.

Mammosomatotropic tumor (*3*, *32*).—The mammotrope which secretes mammotropic hormone is itself highly sensitive to tumorous transformation. It seems to us that the mammosomatotropic tumor is the most common type of pituitary tumor in both mice and rats.

Several investigators, well over two decades ago, first induced pituitary tumors by prolonged treatment of mice and rats with estrogens (*26*). Our attention was called to this when we noted the development of pituitary tumors in irradiated mice. Pituitary tumors occurred 5 times as often in females as in males, and about half the tumors were associated with mammary-gland stimulation (*32*). This relationship led us to repeat the induction of pituitary tumors by estrogens and to inquire into their characters. When transplanted, these tumors had two outstanding effects: (*a*) marked stimulation of the mammary gland, and (*b*) marked enhancement of body and organ growth (*3*, *33*).

More recently spontaneous pituitary tumors of the rat have also been investigated and found to be similar to the estrogen-induced tumors (Kim, U., Clifton, K. H., and Furth, J.: To be published). There has been a parallelism between growth promotion and mammary-gland stimulation by this type of tumor, be it spontaneous or estrogen-induced. The relation of these two activities is our current problem under investigation.

Estrogen apparently stimulates the so-called acidophiles of the pituitary. Tumor development begins diffusely in acidophiles which become

enlarged and coarsely granulated or lose granulation altogether. These changes occur in the course of treatment with estrogen within 30 to 40 days of a 9- to 14-months' incubation period (*34*). Although they are usually described as chromophobic, primary tumors contain 15 to 50 percent sparsely to well-granulated acidophiles (*34*). Their character was first suggested by Zeckwer (*35*) and LaCour (*36*).

Acquisition of autonomy is usually heralded by the appearance of giant cells with enlarged, pleomorphic nuclei, often with inclusion bodies, and an increase in mitotic figures, many of which are abnormal. They occur in scattered nests which often vanish on subpassage. These giant cells are probably polypoid and not viable. It has not been difficult, however, to distinguish morphologically between dependent and autonomous variants.

Farquhar (*37*) compared the electronmicrographic pictures of acidophiles of lactating rat with neoplastic mammotropes and found them to be basically alike. The most distinctive features of these mammotropes are their dense ovoid or globular cytoplasmic granules. They are larger than those of other types of pituitary cells (350–600 mμ). Whether the large and small granules are variants of the same secretory granule, or represent granules containing two distinct hormones, somatotropins and mammotropins, respectively, remains to be determined.

The mitochondria are relatively pale; the endoplasmic reticulum is abundant, varying from tiny ovoid profiles to the parallel rays characteristic of normal acidophiles. In general, Farquhar noted that greater autonomy and loss of functional activity were associated with progressive loss of identifying features. In tumorous mammotropes there was a great variability in cell size and in the form of cellular components. Complicated nuclear infoldings and aberrant mitochondrial forms with randomly arranged internal crests were frequently seen (*37*).

Pituitary tumors in pituitary grafts.—An interesting recent observation is the induction of pituitary tumors by grafting pituitaries of mice in histocompatible hosts. Mühlbock and Boot (*31*) implanted subcutaneously 20 to 200 pituitaries per mouse at weekly intervals. Some grafts gave rise to tumors which resembled spontaneous chromophobe adenomas as well as the tumors induced by long-term treatment with estrogens. In our opinion the cells giving rise to these tumors are the mammotropes.

The pathogenesis of these neoplasms remains obscure. Transplantation, notably when as many as 20 times the volume of a normal organ is involved, can cause several changes and involve the introduction of latent neoplastic cells.

Transplantation studies of others have shown that the pituitary cell producing luteotropin (prolactin) functions in extracranial location (*38*).

Leydig's cell tumors of the testis.—The role of estrogens in induction of testicular tumors and on their progression was studied by Andervont

et al. (*6*). When pellets were removed from mice with small testicular tumors (measuring 10–12 mm. in length), about one half the tumors regressed while the remainder continued to enlarge. When mice, in which the testes had receded to normal size, received a second pellet, the tumors recurred promptly.

When those mice, whose first pellets were removed 5 to 9 months earlier, received a second pellet, one half developed tumors within 4 months. Controls receiving first pellets had a tumor incidence of only 7 percent within 4 months.

The testes of some mice, whose pellets were excised 10 to 16 months earlier, were examined microscopically. Areas of interstitial cell hyperplasia were found in many. Testicular tumors arose in some mice from 1 to 12 months after removal of pellets in absence of testicular enlargement.

These observations indicate persistence of interstitial cell changes initiated by diethystilbestrol, and suggest that these changes are irreversible but in most instances insufficient for further progression unless a second hormonal stimulus is applied.

These observations also suggest that estrogen dependence of induced testicular tumors is limited; progression to autonomy can readily occur when estrogen is removed from hosts carrying primary tumors or when the tumors are carried in serial passage in estrogenized hosts.

Andervont *et al.* (*6*) surveyed the literature and concluded that, prior to their work, a total of 21 Leydig's cell tumors had been transplanted, 11 of which grew in estrogenized hosts only, and 1 of which grew in untreated mice of both sexes. Nine failed to grow in normal hosts, *i.e.*, they were also dependent. On serial passages, estrogen-induced testicular tumors invariably became autonomous.

In the experience of Andervont *et al.*, hormone-dependent and autonomous tumors were histologically indistinguishable.

Other tumors induced by estrogen.—Estrogen-induced cervical and uterine carcinomas were studied recently by Gardner (*39*). These tumors do not go through a phase of estrogen dependency. Perhaps this type of carcinogenesis is allied to that of mutagenic hydrocarbons. It is not permissible, however, to accept this simple explanation without supporting evidence. With respect to leukemias, no estrogen-dependent phase was demonstrable in the few tests made by us.

Estrogen-induced kidney carcinomas in hamsters were entirely dependent on estrogens for 14 serial passages (*15*). This is a remarkable example of the broad capacity of this hormone.

The experience of Alexander and Horning (*40*) with induction of sarcomas with insoluble plastic films should serve as a warning that "innocuous" substances may of themselves be carcinogenic.

Mode of estrogen action.—Among the hormones related to tumors, estrogens are outstanding. Several estrogen-induced tumors have gone through phases of dependency; hence this hormone is eminently suited

for the study of cellular changes leading to tumor formation. The effects of estrogen on synthetic processes, surveyed by Villee (*41*), are listed in table 6. Two hypotheses have recently been proposed to explain these effects:

TABLE 6.—Effects of estrogen on synthetic processes

Uterus		Effect on other tissues
General effects	Increase in synthetic systems	Enzyme increase
Hyperemia		TPNH–DPN* transhydrogenase
Inhibition of water	Rate of incorporation of glycine, alanine, serine, lysine, and tryptophan into proteins	β-Glucuronidase
Increase in rate of utilization of O_2 and P	Rate of synthesis of cholesterol	Fibrinolysin
Increase in dry weight	Rate of incorporation of serine, glycine, and formate into adenine and guanine	Alkaline glycerophosphatase
Increase in ribonucleic acid (RNA), deoxyribonucleic acid (DNA), and protein	Rate of fatty acid synthesis	Arginase

*Reduced triphosphopyridine nucleotide-diphosphopyridine nucleotide.

(*a*) Estrogens activate a number of synthetic enzyme systems (*42*). The nature of such activation is obscure; it may be an "unmasking" of enzymes or increase in permeability of subcellular structures to substrate, or "electrostatic effects analogous to changes taking place in nerve cell membranes" (*42*). Accordingly, tissue specificity is due to quantitative differences between sensitive and nonsensitive tissues in a number of synthetic enzyme systems.

(*b*) The primary site of action is on a specific TPNH-DPN transhydrogenase, linked to isocitric dehydrogenase. This has been found only in estrogen-sensitive tissues (*41*).

There are two postulates as to the exact role of estrogens in this system: Talalay *et al.* (*43*) hold that they act as hydrogen carriers, and Villee (*41*) that they are bound directly to the enzyme molecule, thereby activating it. Estrogenic effects (table 6) require energy, *i.e.*, are endergonic. This postulate presupposes that, as concerns estrogen effect, available energy is *the* limiting factor in end-organ growth, differentiation, or secretion. As more utilizable energy is obtained by oxidation of DPNH than TPNH via the cytochrome system, a transhydrogenase (yielding DPNH from TPNH) would increase energy to the critical level (*41*). The increased energy could, in part, be utilized for synthesis of more of the transhydrogenase and thus the sensitivity of the tissue to estrogen would be increased.

Further comments on progression from normal to autonomous neoplasms.—Talalay *et al.* (*43*) conceive steroid-dependent tumors as those whose growth is limited by steroid-mediated transhydrogenations. Some

rise to a population of tumor cells which can regress when the estrogen is withdrawn (*47*). The situation is similar with thyroid tumors resulting from thyrotropic hormone stimulation (*48*) and with thyrotropic tumors resulting from thyroidectomy (*49*).

Sustained administration of estrogens may increase the quantity of specific energy-yielding enzymes. This may go with an increase in the RNA related to enzyme production by a kind of adaptative mechanism which, in a manner similar to that of antibody formation by lymphoid cells, can be sustained.

It now seems to us that even the fully hormone-dependent tumor cells are modified normal cells, for the following reasons: When hormone-responsive cells have been treated to a degree which would not yet yield a tumor, and the treatment is discontinued and reinstituted at a later time, the cell can rapidly give rise to a neoplasm. *e.g.*, Andervont *et al.* (*6*). Thus, such conditioned cells have a memory. Similarly, established dependent tumors, when stimulated, grow at a faster rate than corresponding normal cells. Dependent tumors are not static. They usually gain in "growth vigor" in the course of successive passages in conditioned hosts and lose secretory capacity while retaining transplantability. The gradualness of increase in growth vigor of tumors was one of the arguments of Loeb against the somatic mutation theory (*50*). Highly dependent cells, *e.g.*, those of thyrotropic tumors, readily grow in extracranial location, while their normal counterpart requires direct connection with the hypothalamus. Whether normal thyrotrope, *i.e.*, pituitary grafts, will yield a tumor in athyroid hosts is currently under study. A debatable point is that, according to data in the literature, the only pituitary cell capable of full function in extracranial location (*38*) and of giving rise to tumors (*26*, *31*) is the mammotrope.

Thyrotropes and thyroid tumors.—Another feed-back system, highly suitable for the study of tumorigenesis by disturbance of homeostasis, is that of the thyrotrope-thyroid system. At the 25th anniversary of the Jackson Memorial Laboratory, the then novel thyrotropic tumor was discussed (*53*). Now, we shall discuss thyroid tumorigenesis and correlate the two.

The precise operation of this feed-back system is not known. There is evidence that thyrotropin acts by direct contact with the thyroid cell (*54*), but not that thyroid hormones inhibit the thyrotropes by direct contact. Now that these hormones and their products can be tagged with radioisotopes and isolated cells of both thyrotropes and thyroid epithelium are available, this feed-back problem is ripe for concentrated exploration. In contrast to the estrogen-mammotrope system, we are dealing here with a situation in which the target hormone, the thyroid hormone, or its product regulates the pituitary by inhibition. Therefore, it is possible to produce either thyroid tumors or thyrotropic tumors at will by rearrangement of the homeostatic system.

cells, however, might possess alternate steroid-independent, enzymatic mechanisms which would bypass the rate-limiting function of the steroid dehydrogenases. By a process of selection, those cells might survive preferentially and give rise to hormone-independent tumors. The question remains whether the cells initially possess this alternate enzymatic mechanism or whether it is environment-induced.

Although perpetuation of a new cell type need not come from genetic modification, the chromosomal changes described in many autonomous tumors (*44–46*) strongly suggest that the autonomous transformation is associated with chromosomal alterations. Cells are constantly subject to mutations. The host selects the more rapidly growing variant or mutant, *i.e.*, the one unrestrained by homeostatic forces. We suppose that both possibilities, adaptative modifications and genetic mutations, are possible.

Whether the development of autonomy is merely a continuation of the same adaptative process leading to development of dependent tumors, or a radical departure from the normal type, perhaps a chromosomal mutation, is now readily amenable to experimental analysis with the modern tools of cytogenetics (*44*).

Is it conceivable that, when "choked" with estrogens, some cells become altered by a mechanism similar to development of resistance to injurious agents, *e.g.*, antimetabolites? Law (*51*) discusses the mechanisms by which such a resistance might be acquired.

The stimulated mammary gland is both an excretory and a secretory organ which may trap carcinogens (*52*). If this is true, the hormones involved are not carcinogens but merely stimulants of certain cells. In addition, stimulated, *i.e.*, proliferating, cells are more sensitive to carcinomutagens, such as ionizing irradiations and carcinogenic hydrocarbons, than resting cells. The mammary gland is rudimentary, virtually nonexistent, until induced to develop by hormones. Estrogens, mainly via the mammotropes, cause the rudimentary duct cells to proliferate and differentiate into secretory alveoli, presumably with acquisition of new enzyme systems but with retention of the basic genic feature of the cells of origin.

The nuclear abnormalities noted suggest that a cytogenetic modification occurs when differentiated mammary-gland cells become autonomous. Yet there is no solid proof as to the primacy and specificity of this change, as most geneticists are inclined to postulate.

Studies on the fate of estrogens in the cells which they stimulate may contribute to the solution of this problem. The overloading of cells with estrogens that occurs in the tumorigenic process deserves special study. A physiologic agent, when applied in large amounts, may become pathologic ("pharmacologic"), provoking changes on molecular levels not caused by physiologic doses.

On the biological level, an essential feature of estrogen action is its reversibility. The mammotropes, when stimulated by estrogens, give

Induction of such tumors in mice is but slightly modulated by genetics and appears to succeed in almost every animal, whereas derangement of the estrogen-mammotrope-mammary-gland system results in tumor formation in either the mammary gland or the mammotrope in both mice and rats. Derangements of the thyroid-thyrotrope system readily yield tumors in the thyroid glands of mice and rats, but only in the thyrotropes of mice (*53*, *55*). Thyrotropic tumors have thus far not been induced in rats. Failure of rat thyrotropes to proliferate continuously remains to be adequately explained. One plausible assumption is that extrathyroidal synthesis of thyroid hormone or a related compound is sufficient to inhibit the thyrotropes. Inherent genetic differences in cell longevity and in capacity of thyrotropes of rats for rapid mitoses may also be important (*26*).

Sustained thyroid-hormone deficiency induced by any means (best accomplished by radiothyroidectomy) will inevitably lead to development of thyrotropic tumors in mice. Almost all first-generation grafts studied have been completely dependent on thyroid-hormone deficiency; *i.e.*, they could be grafted only on radiothyroidectomized animals (*53*). Some strains remained fully dependent for several generations before they gave rise to autonomous variants. These grafted, dependent tumors attained great size and even metastasized to lymph nodes. These observations have some timely implication and will, therefore, be more fully described. Whenever dependent thyrotropic tumors were grafted on thyroidectomized animals, normal siblings also received grafts and were observed for over 1 year. An excellent indicator of the origin of an autonomous variant is the host's thyroid, which becomes enlarged even when the first autonomous thyrotropic tumor measures but a few millimeters. In fact, such tumors have often been missed on gross examination and disclosed only on serial section of the graft site. So, when after a year the graft is not found in a normal host and the animal's own thyroid is normal, it can be presumed that there were no autonomous variants among the grafted thyrotropes. These data suggest that the acquisition of autonomy, which occurs usually in the II to IV passages, is best interpreted as new formation of autonomous cells in the progeny of dependent tumor cells and not as selection of such cells in the grafted population.

The thyroids stimulated by autonomous, highly functional thyrotropic tumors are tremendously increased in size and studded with numerous tumorlike nodules, some of which are interpreted by others as carcinomas. They can metastasize (*56*). Nevertheless, such thyroid tumors could not be grafted on normal hosts. This leads us to problems of the genesis of thyroid tumors.

The experimental induction of thyroid tumors by low iodine diet (*9*) and by antithyroidal drugs (*10*. *11*) is now common knowledge (table 1), and mediation of this tumorigenesis by thyrotrope has long been assumed. Ample evidence has been presented that thyroid-tumor induc-

tion by low-iodine diet or goitrogen follows lowering thyroid-hormone levels, with resultant stimulation of thyrotropic-hormone secretion. Indeed, prevention of the thyroid adenomas and carcinomas in man, in regions where the water is deficient in iodine, is one of the greatest discoveries in cancer prophylaxis, and should be cited as an example of how knowledge in homeostatic control can lead to prevention of human cancer. It has also been assumed that excess of thyrotropic hormone will induce thyroid tumors. To prove this, several associates have attempted to induce thyroid tumors by sustained excessive stimulation with this hormone. Commercial thyrotropic hormone is toxic in large quantities. Endogenous thyrotropic hormone can be supplied in large quantities over long periods by grafting highly functional autonomous thyrotropic tumors in one thigh of intact mice; such treatment maintains the growth of dependent thyroid adenomas in the other thigh. Ghera *et al.* (Ghera, N. H., Pullar, P., and Furth, J.: To be published) succeeded in developing fully dependent thyroid tumors.

Table 7 shows the behavior of such tumors in successive passages. They grow equally in normal and athyroid hosts. Figure 7 shows a solid adenomatous lesion in a thyroid that was stimulated by thyrotropic tumor. Figure 8 is such a lesion grafted in another thyrotropic tumor-bearing mouse. This would probably be diagnosed as a cancer, but this tumor does not grow on normal mice. Papillary and alveolar "carcinomas" of thyroid in humans that can be well controlled by thyroid hormone are probably of this type.

TABLE 7.—Results of transplantation of thyroid tumors in normal and conditioned hosts

Line	Passage	Time of thyrotropic-tumor graft	Treatment of host			
			Bearing thyrotropic tumor		Without thyrotropic tumor	
			Normal	Athyroid	Normal	Athyroid
34	I	Simultaneous	3/4 (270)*		0/5	
	II	Simultaneous	5/6 (250)	3/5 (310)	0/5	0/5
	IIIA	Ninety days earlier	8/8 (60)		0/8	
	IIIB	Simultaneous	7/8 (218)	2/8 (282)	0/8	0/8
	IV	One hundred-thirty days earlier	4/12 (62)†	0/17†	0/6†	0/6†
Total			27/38	5/30	0/32	0/19
8	I	Simultaneous	2/3 (325)		0/6	0/6
	II	One hundred-nine days later	5/6 (326)	2/4 (376)		0/9‡
Total			7/9	2/4	0/6	0/15

*Number of animals with tumors over number grafted. Average latency, in days, is given in parentheses.
†Animals without tumors still alive.
‡Animals given propylthiouracil.

In the course of passages, such tumors slowly gain in growth vigor though they retain dependency. When these thyroid tumors reach a

few centimeters in diameter in intact hosts, the animal's own thyroid tissue is only moderately enlarged. This, with retention of dependence on the normal stimulant, is a neglected feature of dependent tumors; it indicates that dependent tumor cells are altered and that the alteration is progressive.

Study of such tumors calls for long observation because of their slow growth. The following simple device can be used to hasten growth. The autonomous thyrotropic tumor (the source of thyrotropic hormone) is grafted on the animal long before the thyroid tumor so that, at the time of the latter graft, the host is loaded with circulating thyrotropic hormone (*57*). In such an animal, a dependent thyroid tumor appeared within 46 to 62 days, whereas in those with mild thyrotropic stimulation, the latency period of the tumor exceeded 200 days.

Experiments have been started on isolation of the organelles of the monomorphous experimental pituitary tumors, in which these hormones are produced by means of cell fractionation technique, and attempts are being made to produce these hormones *in vitro* in tissue cultures. Such studies offer an approach for exploration of the *modus operandi* of feed-back systems.

The great problems in tissue culture are (1) how to keep normal cells normal and (2) what the forces are that make cells change *in vitro*. If the assumption is correct that cells proliferate continuously *in vitro* because of lack of specific restraining forces, tissue cultures may be used in search of physiologic inhibitors. In some hormone-producing cells, growth was continuous but hormone production was lacking. Such cultures may be used in the search for functional stimulants. Growth and function need not occur in parallel. The steady proliferation of cells alone enhances the chances for mutation, and the abnormal environment of tissue culture may contain carcinogenic agents. Attempts to induce neoplastic transformation of cells *in vitro* with known carcinogens have, however, been disappointing. Only viruses have been shown to be capable of accomplishing this (*58*).

Viruses as conditioning agents.—This survey has thus far covered only experimental tumors and only hormones as conditioning agents.

It should be mentioned here that viruses resident in cells can also condition genetically unaltered cells to seemingly autonomous growths (*59*, *60*). This was first proposed by Rous (*61*). Recently the number of proven virus tumors in animals has mounted. None is yet known in man, despite 40 years of intense research in animal tumor viruses, but techniques suitable to tackle the human problem have not been known until recently. Now that suitable techniques are available, the discovery of some virus-conditioned neoplasms in man may be simply a matter of time.

The concept of virus-conditioned neoplasia is not widely accepted. There are several mechanisms by which viruses resident in cells can break the homeostatic control of cell growth (*59*, *60*). Those mecha-

nisms which do not involve a hereditary modification of the cell genome can be properly related to conditioned (reversible) growth disturbances. The recently discovered virus of Friend (*62*) is a good example (*63*). Levan (Discussion at the Conference of the New York Academy of Science on Subcellular Particles in the Neoplastic Process, October 1957) found no chromosomal change in the Friend leukemia cells. In our experience these cells, when introduced in susceptible hosts, usually do not proliferate but yield the virus which stimulates reticulum cells throughout the spleen.

Two viral neoplasms of mice, studied by us currently, are other good examples. One is thymic lymphoma induced by Gross virus (*64*, *65*), the other a reticulum-cell sarcoma induced by Friend virus (*62*). The reticulum-cell sarcoma lodges in reticular cells of the spleen and drives them to unrestrained proliferation. This hyperplasia is recognizable as multicentric foci of a uniform population of cells lacking the features of anaplastic cancer. Only after numerous unsuccessful attempts were transplantable cells obtained (*66*).

The salivary-gland tumors of mice induced by polyoma virus are another example of virus-conditioned neoplasia (Buffett, R. F., and Furth, J.: Unpublished data). Grafts of these tumors seldom succeed in histocompatible hosts. In these tumor cells the virus is carried in "masked" form.

Under the conventional microscope, the virus-carrying mammalian neoplasms differ in no essential way from common cancers which are thought to be virus-free, be they leukemia, carcinoma, or sarcoma.

Histologic characteristics of tumors.—Identification of tumors as dependent or autonomous in the original host is a distressing topic. A recent annual symposium (1959) of the American Association of Pathologists and Bacteriologists underlines our ignorance concerning human tumors. Even in the domain of animal tumors the observations are sketchy and unreliable.

Our goal is clear: We aim to identify biopsied specimens as to tumor type, dependent or autonomous, and to determine the stimulating and restraining forces of given tumor-cell populations. The fully dependent and highly autonomous experimental tumors studied by us are markedly different in appearance. The dependent tumors resemble a population of normal cells; the autonomous tumors have the characteristics of anaplastic carcinomas (*56*).

Consultation with several colleagues during the past years and a review of some of their clinical material, however, have convinced us that, in practice, such a morphological characterization is not possible. But we are also impressed by the scarcity of investigative efforts to obtain and study the proper specimens.

There are many conceivable avenues for future research other than morphology. Tumor fragments should be submitted to a thorough quantitative histochemical and functional assay. There are some frag-

mentary studies in this direction; *e.g.*, Reynolds (to be published) compared various experimental thyrotropic pituitary tumors and found a positive correlation between the degree of responsiveness on the one hand and O_2 consumption, aerobic glycolysis, lactic acid production, and the degree of morphologic dedifferentiation on the other. Cohen *et al.* (*67*) incubated adrenal tumor slices of mice and rats with corticotropin (AtH). These tumors caused Cushing's syndrome *in vivo* (*68*). *In vitro*, the tumor slices secreted corticoids in proportion to the added AtH. The steroids were identified not only in character, but a change in their spectrum was discovered in the course of "dedifferentiation" (Cohen, A. I., and Bloch, E.: To be published). Correspondingly, Cushing's pituitary tumors can be assayed *in vitro* by the ability to stimulate the production of corticoids (*69*).

It is conceivable to apply such techniques in search for substances which specifically inhibit a given tumor.

Dominguez *et al.* (*70*) studied the function *in vitro* of Leydig's cell tumors of mice, and Engel studied ovarian tumors of women (*71*). The trend is to identify the enzymes in the tissue that perform certain steps in steroidogenesis.

Conceivably, mammary tumors could be assayed by incubation *in vitro* with mammotropic hormones, which detects stimulation by heightened anabolism, perhaps by as simple a procedure as measuring uptake of labeled amino acids.

Likewise, if the thyrotropic hormones stimulate the thyroid, and androgens the prostate gland by direct action, it may be possible to devise methods *in vitro* which may indicate specific stimulation of their tumors. The choice of the endpoint is up to the imagination of the investigator. It could be a hormone itself, which, as thyroid hormones, can be chromatographed, or a specific enzyme, as acid phosphatases of the prostate, which can be measured chemically and histochemically.

Another conceivable assay is immunologic. Fluorescent antibody technique (*72*) may prove particularly useful for localization of biologically active substances. Protein hormones are antigenic, and steroid hormones, as Lieberman *et al.* (*73*) have shown, can be made antigenic.

Discussion.—Until recently the concept was prevalent that the cancer cell is a permanently altered cell akin to a somatic mutant, *i.e.*, the basic alterations residing in the chromosomes; a minority of investigators assumed infective viruses to be the main if not sole cause of cancer.

When in recent years both chromosomes and viruses were found to be composed of nucleoproteins (NP) with the genetic information residing in nucleic acids (NA) and the proteins acting as vehicles, the cancer problem became restated as one of NA. (Dissenting minority views such as those relating the prime cancerous change to respiratory granules have now been almost abandoned.) This concept enables logical unification of the two competing ideas as to the basic cancerous

change: somatic mutation and viruses. Bacterial viruses (bacteriophages) have been known to be capable of injecting genes into the genome of cells or changing given heritable characters of cells by introduction of noninfective NP. Since the unit components of NA are few, genetics has been more clearly defined as being related to the structural sequence and relationship of the unit components. According to this concept, cancer originates suddenly as a consequence of alteration in NA brought about by carcinogenic chemicals or radiation, or from derangement of the genome by introduction of foreign NA.

We believe this concept of DNA change to be true for most autonomous cancers. It has two disturbing correlaries: (1) It does not explain the mechanism by which the alteration is brought about. (2) It confronts us with a nasty end product: The autonomous cancer cell, which, in spite of heavy investments, has proven to be little responsive to corrective measures. Research on the origin of cancer cells is of greater promise for finding corrective measures.

Those who, as Rhoads (*74*), consider modification of DNA as the nucleus of the cancer problem begin the "*Ring*" with "Götterdammerung," the autonomous villain. We propose to focus attention on the origin of the neoplastic state: *i.e.*, to study the mechanism by which normal cells can pile up to form tumors without a change in DNA, and to study the state in which the apparently autonomous variants arise and the mode of their origin.

The evolution of many cancers has been clearly traced to a gradual or sudden derangement of the physiologic homeostasis. The change from normal-to-dependent and from dependent-to-autonomous cell type can be concisely stated only in terms of physiologic behavior. It occurs in the natural tumor host and is not related to isoantigenic modifications which are linked to surface antigens.

Thus far the problem of change from normal to dependent and from dependent to autonomous has been treated only on a population level. At this level it lacks the suddenness of mutation. However, the offspring of even a single cell can give rise to different clones, well-illustrated in the tissue-culture studies of Sanford (*75*) who noted the development of several neoplastic clones in the progeny of a single normal cell.

Reasons are thus advanced for the view that even fully dependent tumors are composed of altered cells, in which the change does not affect a single fundamental characteristic, *i.e.*, they are homeostatically controlled. However, dependent cells gain proliferative vigor in the course of successive transplantations. The latter applies to autonomous tumors which, with rare exception, gradually lose responsiveness to the forces normally regulating cell number. A precise analysis with a fixed number of cells for transplantation with tests for clonal homogeneity of the population has thus far not been made. What now appears to be *gradual* on a population level may turn out to be *sudden* on a cellular

level. This sort of analysis may yield a lead as to whether the somatic heritable change is truly genetic or whether it is a self-perpetuating disturbance with environment-induced quantitative changes in enzyme patterns without genetic alteration. The closest analogy of the latter would be differentiation.

Are hormones carcinogens?—Hormones are best conceived as regulators of cell growth and not as carcinogens. They enhance the likelihood of cancerous transformations, however, by driving cells to multiplication. We accept the axioms that (1) cell multiplication enhances the likelihood of autonomous transformation, and conversely, that putting cells at rest diminishes it; and (2) even after cells are "initiated" (by this we mean a change in character of their hereditary units), secondary stimulants determine the very occurrence of the autonomous transformation. Herein lie possibilities for cancer prophylaxis.

Radiation mutagens may not be all evil.—Natural mutations are looked upon by cancerologists as bad, since they give rise to new cell types from which the body selects only the worst. Mutation can also be looked upon, however, as the introduction of new useful types. Unless there is inheritance of acquired features, the mutagens occurring in nature are the main dynamic creative force of evolution. Without such opportunity life would be static.

Background radiation has been looked upon as a Mephistopheles—doing only evil, causing cancer, and shortening the lifespan. Botanists, however, have learned how to make use of ionizing radiation to create better plants. Perhaps instead of worrying about such mutagens, which are an inevitable part of life, we should inquire as to how we can make the best of them.

Closing plea.—In these days of concentration on the noble purpose of killing the autonomous cancer cell, our plea is for intensification of research on the origin and nature of the neoplastic transformation.

To abstract scientists, the studies here reviewed point to a means of securing, for basic research, normal and homologous cancer cells with varying grades of deviation from normal. To the practical-minded they are the key to the prophylaxis of many common cancers, such as those of the mammary gland and endocrine organs.

To pathologists, the discovery of dependent neoplasms and the responsiveness of autonomous tumors represent a unique challenge. The century of Virchow gave us the doctrine of cellular pathology and the recognition of neoplasms by morphologic identification of changes in cells and in cell populations. The tools of pathology have since been the microscope with the immortal hematoxylin and eosin. Now we recognize that morphology alone is inadequate to characterize tumor cells. Scientists of different disciplines must join forces. Cancer scientists must first learn about functional and biochemical alterations of living cells to detect and characterize tumor cells, even in absence of morphologic changes. This will lead to an understanding of the pathogenesis

of neoplastic diseases, and possibly to the control of them while they *are* controllable.

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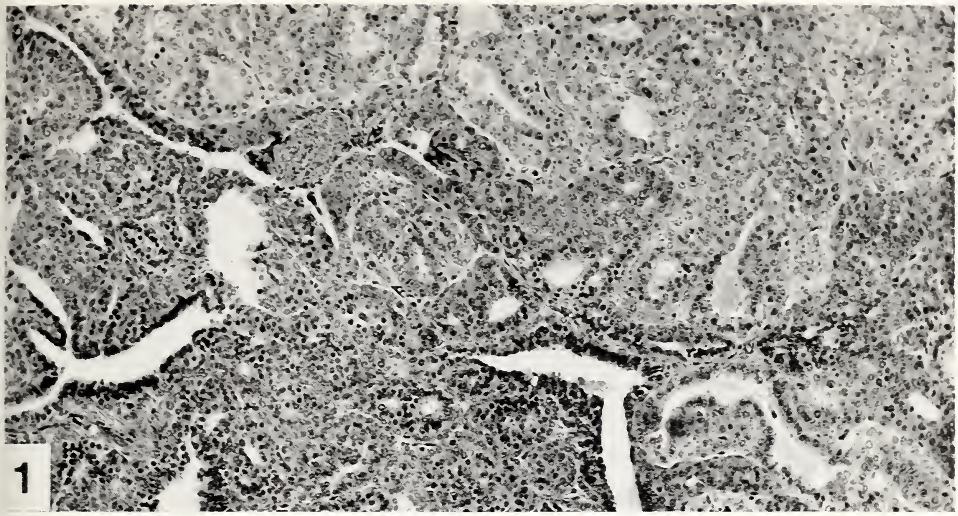
PLATE 7

FIGURE 1.—Primary mammary adenocarcinoma (HMT-F2) in female rat that had received 290 mg. methylcholanthrene over 19 weeks. Treatment was started when rat was 2 months old and tumor was first palpated 3 months later. Hematoxylin and eosin. × 300

FIGURE 2.—Fibrous tissue and pyknotic epithelium in graft of methylcholanthrene-induced mammary tumor (HMT-F2) in untreated male rat. Tumor nodule measured 2 mm. in diameter at death, 3 months after grafting. Hematoxylin and eosin. × 300

FIGURE 3.—Graft of same tumor as in figure 1 in intact male rat that had also received a graft of mammotropic tumor (MtT) 3 weeks before death. Mammary tumor measured 2 mm. in diameter at that time. When animal was killed at 5 months of age, mammary tumor measured 23 mm. in diameter, and MtT 20 mm. Hematoxylin and eosin. × 300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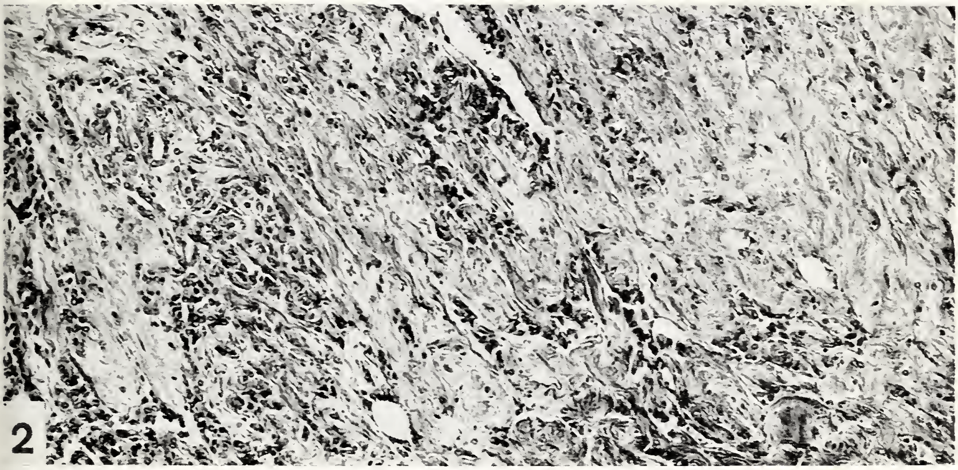
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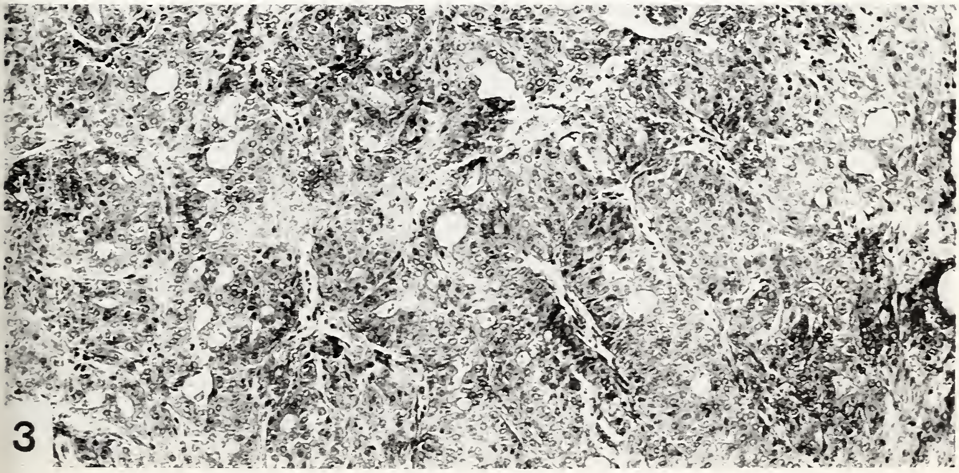
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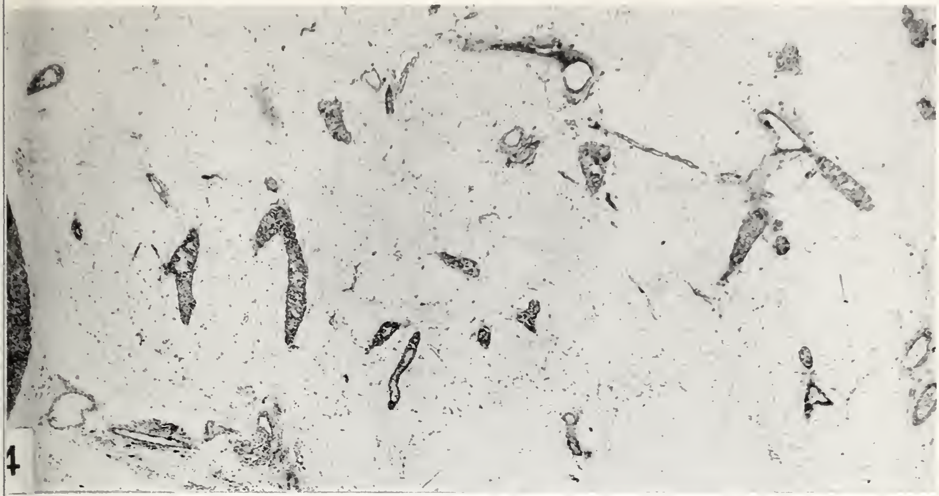
PLATE 8

FIGURE 4.—Mammary gland of hypophysectomized 6-month-old female rat implanted with pellet containing 3.5 mg. diethylstilbestrol, 5 months before death. Two weeks after implantation, this pellet was replaced by one containing 1.5 mg. stilbestrol. Hematoxylin and eosin. × 50

FIGURE 5.—Proliferation of mammary-gland epithelium with secretion. Age and treatment of this female rat were the same as in figure 4; this animal, however, bore a mammotropic tumor (MtT) measuring 12 mm. in diameter, 4 months after grafting. Hematoxylin and eosin. × 50

FIGURE 6.—Adenocarcinoma *in situ* in highly stimulated mammary gland of 5½-month-old intact female rat. The rat bore an MtT measuring 35 mm. in diameter, 2½ months after grafting. Hematoxylin and eosin. × 50

Figures 4 and 5 are reproduced from *Endocrine Aspects of Breast Cancer* by C. F. W. Illingworth, by permission of E. & S. Livingstone, Ltd., Edinburgh.



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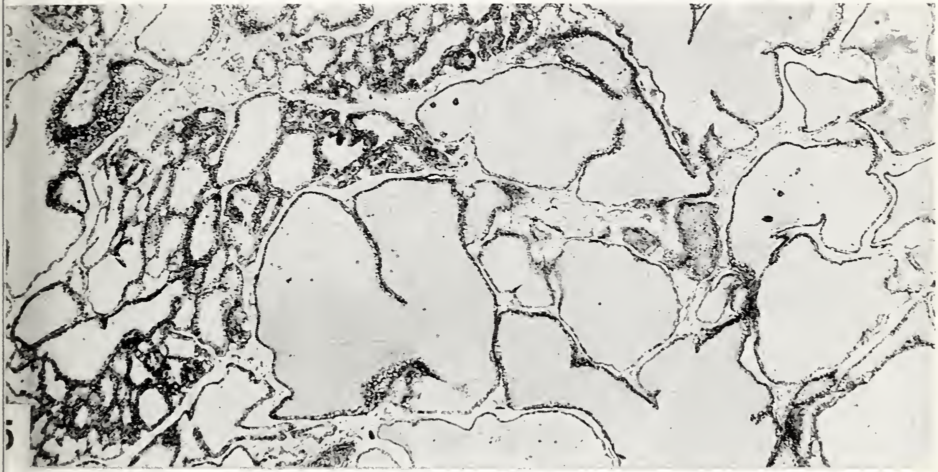

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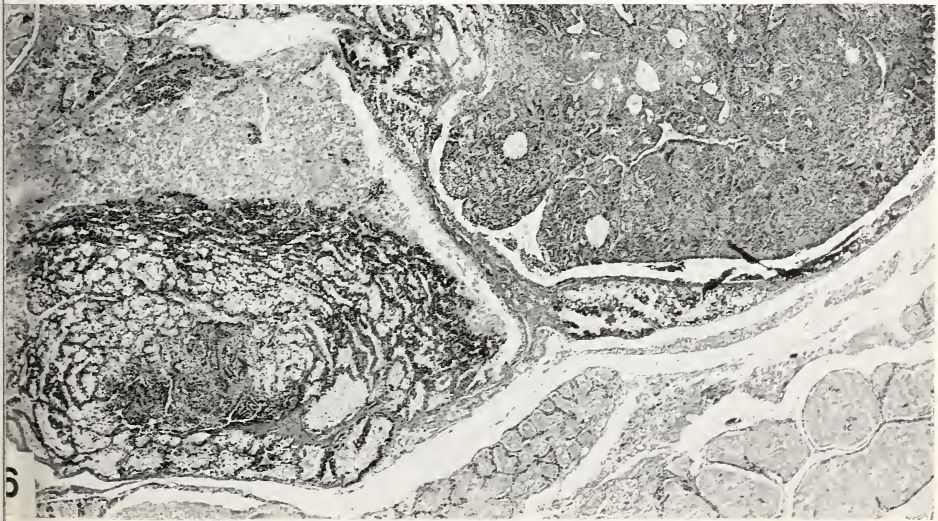

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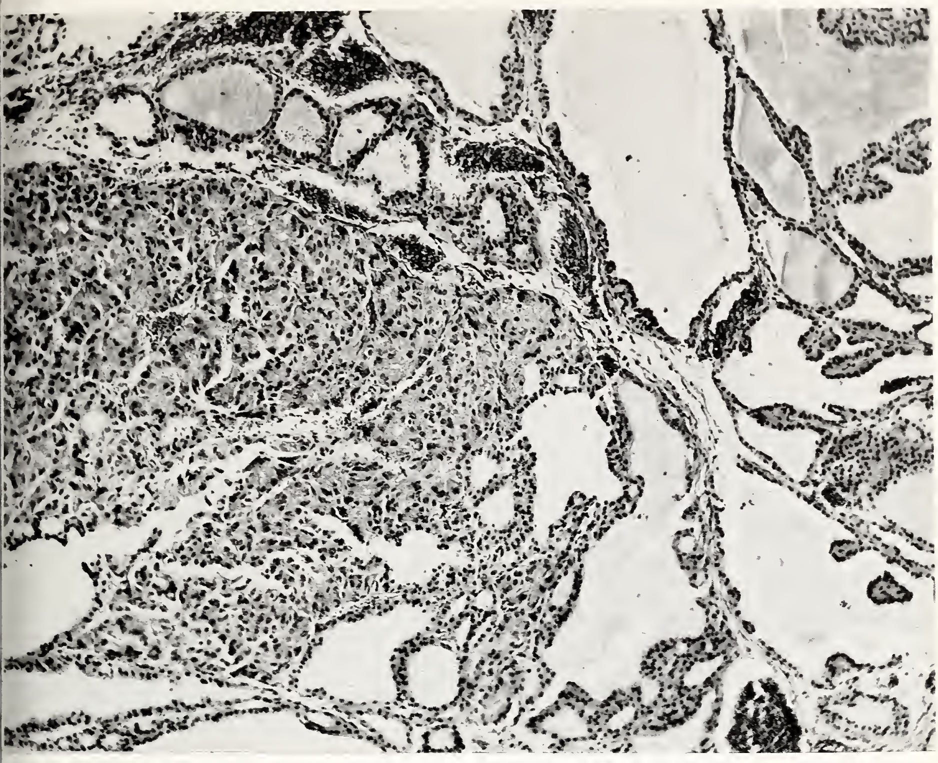
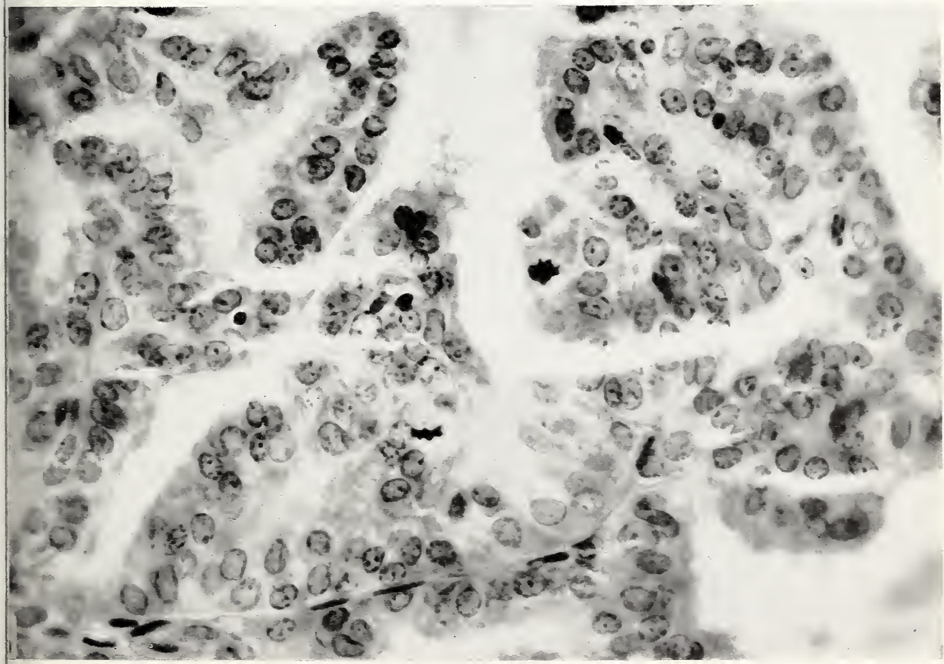
PLATE 9

FIGURE 7.—Thyroid stimulated by thyrotropic tumor (TtT). Section is taken through a predominantly solid area. Hematoxylin and eosin. × 160 (Ghera *et al.:* To be published.)

FIGURE 8.—Transplanted thyroid adenoma similar to one above in TtT-bearing mouse. Higher magnification showing cellular pleomorphism and mitotic figures. × 625 (Ghera *et al.:* To be published.)





Prospects for a Systemic Therapy of Cancer[1, 2]

A. M. Brues, *M.D., Division of Biological and Medical Research, Argonne National Laboratory, Lemont, Illinois*

Those who arranged this excellent symposium appear to have fallen into an error which is becoming all too common in modern scientific communication—that of equating one scientist with another, or, in other words, assuming that an authority in one matter can speak with authority in another. With such transduction of authority, a person is likely either to oversimplify grossly the subject or to stand in awe before its inscrutability and complexity. Then, too, this is a subject fraught with emotional content for the investigator who, often implicitly, has been aspiring to a fortunate solution to the whole problem, and for a public that has seen the subject in an aura of fear and hope.

My own interests have been primarily directed toward appreciation of the genesis and nature of cancer rather than toward its cure, and I have tended to believe that its conquest is more likely to be secured through comprehension of the nature of the process than through research *ad hoc*. Probably I am biased in this matter because the study of the true nature of cancer is a most fascinating branch of pure biology, with ramifications through all the life sciences. One of the best things that could happen in biology—and I think it is beginning—would be a full-hearted appreciation of the dignity of cancer research and of the need, as a part of basic training, to understand its vast and generally competent literature.

On this basis I consented to speak on the subject of the prospects for a systemic therapy of cancer. Furthermore, I am speaking at the 30th anniversary of the Roscoe B. Jackson Memorial Laboratory, which for 3 decades has identified itself with studies of the basic nature of cancer, and of the organism in which it arises as a key figure in the cancer process. Many examples can be given of the indestructible influence of this laboratory on basic cancer research, but I shall not attempt to outline them because of the impracticability of giving everyone due credit. Bridging the gap between virology, cancer, and genetics; the use of statistically useful numbers of mammals in the study of carcinogenesis; immunogenetics; and a willingness to go beyond the sacred tenets of fly genetics are among these. No cancer cure has been provided, yet I am sure that the collective contributions of this laboratory and of its former

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[1] Presented at a symposium on normal and abnormal differentiation and development, held in celebration of the 30th anniversary of the Roscoe B. Jackson Memorial Laboratory, June 15-17, 1959, at Bar Harbor, Maine.

[2] This work was prepared under the auspices of the U.S. Atomic Energy Commission.

staff have contributed far more in that direction than could have been anticipated on any random basis.

I do not mean, by all this, to discount chemotherapy programs. Those of us who are students of cancer have an obligation, as a whole, to leave no stone unturned whenever modern techniques will move it. I might go further to say that the modern chemotherapy program can be less rewarding to the investigator than basic research, but it is to the great credit of those who have conducted these programs (or perhaps it is a reflection of human nature) that they have been conducted with a full sense of serendipity and appreciation of the excitement of the basic and the unexpected. More than that, chemotherapy programs have contributed in a massive and effective way insofar as they have helped pharmacology to escape from the empirical and from emphasis upon neurotoxicology into cytopharmacology, and to appreciate the role of the whole cell and of its critical components in the understanding of drug action.

Some years ago I had a similar position on a cancer symposium: to make a few remarks about the potential approaches to the then embryonic science of cancer chemotherapy (*1*). I recognized at that time that there might be useful approaches through perusal of catalogues of chemical agents, through pursuing studies of modifications of the molecules that have shown promise, through logical processes based on current understanding of cancer, or through pure chance. It was uncertain, and will remain so, which approach will be the most useful. No doubt all of them deserve a share of attention. An example of this is the discovery of podophyllin. In an experiment in which 500 promising classes of compounds were tested methodically, podophyllin was shown to be a carcinostatic agent (*2*). At approximately the same time, in another laboratory, by a wholly chance observation, some tissue cultures quite unexpectedly showed toxic effects. Careful inquiry showed that the human serum used in the culture medium came from a patient who was being treated with podophyllin for venereal warts (*3*).

HISTORICAL

The early history of research in the therapy of cancer is of some interest. While our subject rules out essentially local therapy, this has always been recognized as the most nearly successful mode of treatment, whether it involved surgical removal, ligation of arteries, mechanical compression, the use of caustics and escharotics, or, more recently, ionizing rays. While, in general, topical applications were employed mainly to control unpleasant aspects of ulceration, as in the use of arsenic (Plunket's remedy of around 1800), it was recognized that such applications might produce a "separation of the disease" in which all the diseased parts sloughed off (*4*). Walshe, writing in the 1840's, tells us ". . . upon the incurability of the disease by merely medicinal means almost all writers are agreed; but in respect of the possibility of eradicating it from the system by operation there is no such unanimity" (*5*). In his citations a cure rate of about 5 percent was usually described. In that day, when microscopic evidence of cancer was fully, if primitively, recognized, it was possible to remark concerning the 2-year results on extirpation of mammary cancer reported by Mr. Hill of Chester in 1770—which showed better than a 70 percent 2-year cure rate—that "indubitably" Hill was dealing with a number of noncarcinomatous conditions (*6*). By 1884, with the intervening development of surgery, we read that ". . . the treatment of cancer in the early stages can only be undertaken by the surgeon . . . in the later stages the physician may be called upon to treat symptoms, but up to the present time all the specifics introduced either by general practitioners or by charlatans have proved quite inefficient, if not actually harmful" (*7*).

From that time to now there has been steady progress in the local treatment of cancer, as a result of increasing experience of surgical technique and care, advancing understanding of the natural history of the disease, and improved diagnosis. Radiation therapy, which was already considered a proper procedure in handling superficial tumors 6 years after discovery of X rays (*8*), has shown a parallel steady progress, in which physics has played an additional major role.

Systemic remedies have nevertheless had their vogue, as they naturally would in an ordinarily fatal disease. Without supportive therapy and diet, the longest history of therapeutic use may be attributed to arsenic, which was first recommended in the 10th or 11th century by a monk named Theodoric. One Swedish practitioner of the 18th and 19th centuries claimed to have cured 30 cases of well-marked cancer in the course of 50 years' practice. Others have denied its efficacy, while still others have asserted that it retards progression from the scirrhous to the ulcerative stage. Walshe quotes a description of a case, described by Thompson (*9*), of apparently advanced mammary cancer with edema of the arms, which regressed after therapy with the iodide of arsenic, and makes a comment whose relevance may be paralleled in contemporary scientific criticism: "The number of times leeches were applied is not stated, and the treatment was altogether of too composite a character to allow of our forming a precise estimate of the value of iodide of arsenic." We may at least say that arsenic has had a long history of usage and that until very recently has been employed in the treatment of leukemia: In fact, but for X rays, it would no doubt still be in use generally, and in the process of being gradually displaced by the newer chemotherapeutic ventures. The same might be said of benzol.

Another treatment that had a considerable period of acceptance was the alkaloid coniine, or at least materials of the plant *Conium maculatum*, which was the hemlock of classical days. This was proposed by Störck in 1760 as an "infallible" remedy (*10*). Its infallibility was soon disestablished, yet about 70 years later Recamier affirmed that several cases of both external and internal cancer had yielded to the use of this drug (*11*).

No doubt coniine is but one of many tried (like snake venom) and untried drugs having a palliative effect on cancer symptomatology, and no doubt its vogue for almost a century could be attributed to the enthusiasm, sense of imminence, or press agentry of Störck (factors that still influence the acceptance of proposed therapies for cancer). The fact remains that in recent years, when thousands of compounds have been tested on many times as many test objects for carcinolytic activity, neither coniine, propylpiperidine, nor any of its piperidine analogues has ever appeared in any reported experimental chemotherapeutic venture, from 1900 until the present (*12*). If anyone wonders whether empirical chemotherapeutic research may have run its course, I think this answers the question. Coniine, while still having a place in textbooks of pharmacology, has been the subject of only 3 papers in the last 20 years, and one of these was on the death of Socrates.

Other chemotherapeutic agents appearing in the past literature have run a transient course and probably are not relevant to our problem. Some or most of these have depended upon certain theories of cancer that have been in vogue at one or another time, others to chance observations. A noteworthy observation by Eason in 1793 (*13*) of the disappearance of a scirrhous tumor in the breast after its possessor was struck by lightning, for example, led to widespread use of electricity and galvanism, the possibilities of which were well explored in the ensuing century (*14*).

Finally, I might mention the use of bacterial antigens, such as Coley's toxin and Shear's polysaccharide: Their use arose out of the observation of an occasional human tumor regression when there was also serious infection. There is no question that such materials are at least occasionally successful in inducing a systemic tumor regression. Their use in amounts approaching the effective has been attended with severe toxic symptoms. Because of the high ratio of risk to benefit, the bacterial antigens have not come into general use, though Coley originally believed they might be specific in the treatment of bone tumors (*15*, *16*). It may be useful to consider this fact when claims, not too dissimilar, are made for materials of wholly unknown composition and very costly to prepare (*17*).

BASES OF THE CHEMOTHERAPEUTIC APPROACH

To look squarely at the problem, we must first define cancer. Twenty years ago this was a very simple matter: Cancer was a cell of some particular type that had gone wrong, was autonomous, proliferated indefinitely, and grew rapidly. It was a cell of the host and therefore not subject to its defense mechanisms, nor was it responsive in general to those influences normaly controlling growth of tissues. Much of this is no longer easily believed, and, as we outline the potentialities for a systemic cure of cancer, we have to consider whether cancer may be a single disease (at least in the operational sense of having a single mode of cure) or a conglomerate of many states whose biological behavior has something in common; or even whether, as suggested by Smithers (*18*), we had not best abandon the word as a misleading one and include it under tissue malformation.

The picture that seems to be emerging is that, as a rule, a progressive series of changes takes place in the cells in some area, and perhaps the most critical stage from our viewpoint is the transition from the noninvasive to the invasive tumor. Much can, should, and will be done in the control of cancer before this point, but I will consider the later stages when, presumably, the disease is no longer local. Also, the tumor cell has the potentiality of undergoing further changes on a mutational basis or a dependence on chromosome number and balance, which may cause its descendants to adapt to a new situation like ascites growth, or which may make it resistant to a therapeutic agent. Just at the time when cancer investigators generally have found reasons to abandon the view that a single occurrence suddenly converts a cell into the progenitor of a full-blown malignant clone, some authorities remote from the study of cancer have rediscovered and embraced it (*19*).

One temptation has been to use inhibitors of growth or of cell division in the treatment of cancer. This may be satisfactory locally but should not be expected to be useful as systemic treatment, for the organism itself has a cell growth requirement as stringent or more so than that of the tumor, and this is most critical in maintaining the function of the blood-forming tissues and of the intestinal lining. The failure of total-body X irradiation to act as more than a palliative even in the most radiosensitive cell types suffices to predict many other failures as well. A growth inhibitor that could be induced to concentrate itself specifically in tumor would be another matter, and much of the work with various analogues is encouraged by the fact that some of the analogues do seem to differ in their influence on different tissues.

Some incidental characteristics of experimental tumors have led to therapeutic suggestions: One is the fact that the Shwartzman phenomenon may occur in a tumor without prior sensitization. This seems to be the basis of the bacterial-antigen effect and of the chief action of colchicine on tumors as well (albeit it is best known as a mitotic inhibitor). The concentration of macrophages that occurs around experimental tumors, plus the tendency of the tumor cell to ingest macromolecules, as discussed by Greene (*20*) in another paper on this program —a phenomenon also observed as pinocytosis in tissue cultures—have led investigators to think that one or another colloid, which may be made radioactive, will concentrate in the tumor *per se;* actually, higher concentrations appear in some normal organs laden with reticuloendothelial elements. These special characteristics are most striking in the transplanted tumor, which for reasons of convenience has been widely used in therapeutic studies. The bacterial toxins and colchicine will, for example, eradicate 100 percent transplanted mouse tumors (at least homologous tumors) under suitable conditions, but have not achieved general clinical recognition.

Much of systematic chemotherapy relies on the testing of compounds related to those known to possess some activity, or on a search for analogues of compounds that may be essential in cell building; *e.g.*, pyrimidine analogues. It would seem that insofar as one deals with factors such as radiation, and compounds such as alkylamines, whose main, nonspecific action is to deter growth, the susceptibility of those tissues, whose continued proliferation is necessary to life, will interfere critically with their action against cancer. We must then rely on the fact that tumor metabolism may be obligated to some pathway which normal proliferating cells can circumvent, or we must look to the possibility that some essential principle, regulating cell growth by a feedback mechanism, has been deleted from the malignant cell and, what is more, that this deficiency can be replaced in some way (*21*).

Various compounds, developed through such systematic lines of thought, have been tested and shown to be carcinolytic. Human trials, when attempted, have generally been less encouraging in attaining the goal of a "cure." The development of resistance by the cancer cell is an important factor here, and Rhoads has recently discussed various ways in which this may come about (*22*). The mere fact, now well established by chromosome cytology, that cancer cells may in the course of adaptation to a new environment undergo wide variation and shifts in chromosome number and morphology—something generally forbidden to "nonmalignant" somatic cells—indicates what one is undoubtedly up against here.

Basic to these questions is that of whether there is indeed a fundamental metabolic pattern from which the tumor cannot escape and survive. The most promising possibilities have been and are being actively pursued, and who can say that failure to date is an omen of futility? We must only wait.

Closely allied to this is the question as to whether a compound can become so highly concentrated, or so long retained without metabolic turnover in a tumor which traps it, that a radioactively labeled atom can be made to act as a local carcinolytic agent. I would not want to rule this out, but would only point to the fact that the radiation dose required to cause full local regression of a tumor of median sensitivity is between 10 and 20 times that which the whole organism (and, probably, its blood-forming tissues alone) can survive, so that the ratio of dose-to-tumor tissue to the dose-to-critical normal tissue is inconveniently high. To rationalize this, we can say that an organism, in which the reproductive capacity of the intestinal epithelium or of the blood-forming tissues is reduced to 10 percent, is in a very serious plight, while if all but 10 percent of the tumor cells are sterilized the evil day is only delayed.

The only instance in which localized, self-selective radiation therapy to metastases has successfully been accomplished is that of malignant tumors of the thyroid treated by radioactive iodine. Here we rely on a peculiarity of the parent tissue, which is a still more efficient iodine trap than any cancer derived from it, and on the fact that the thyroid is a dispensable tissue, whose ablation in the early stages of treatment actually helps, either through thyrotropic stimulation or by removal of competition for iodine, in the later concentration of the isotope in tumor cells.

Another class of neoplastic disease, which has shown special sensitivity to chemotherapy in general, is that of the leukemias. These are also notoriously sensitive to radiation, and it may be inferred that they are simply more vulnerable to destruction by agents whose primary action is to inhibit synthesis or to disturb the cytogenetic integrity. A type of approach that will no doubt be useful in handling any therapeutic agents is now under active investigation in conjunction with radiation therapy. It is well known that oxygen potentiates the action of radiation on cells in general and that this can account for some of the vagaries in experimental results of radiation *in vitro*. Any means that will accomplish a differential state of oxygenation of tumor and of critical normal tissues may therefore be used to enhance carcinolysis by radiation. The restorative effect of bone marrow infusions after total-body X irradiation has been used in cases of radiation accidents and may offer some prospects in total-body irradiation therapy of the leukemias, since it enables the organism to survive doses 50 or more percent higher than otherwise. This has opened up a very interesting field in the study of responses to infusion of homologous tissue.

Then there is the general question of whether there can be general therapy for all cancer, or whether many or most tumors will respond differently to several agents. In regard to the "dependent" state in which many tumors of endocrine origin may exist, this has been suspected for a long time and has been especially emphasized by the recent studies of Furth (*23*). While this thought will complicate the screening-program approach, it at least has brought the clinician back into the picture, with added encouragement to exercise scientific insight.

Two additional approaches are beginning to have a serious impact on our thinking, both of them stemming from basic work of the Jackson Laboratory. One is the immunogenetic. Both loss and gain of immunologic identifiers have been observed in tumor cells, and it has even been suggested that addition of some slight incompatibility may be an essential step in the neoplastic procession (*24*). The other is the virologic, in the broadest sense. That new determinants can be introduced directly into the genetic apparatus of the cell has led to much speculation as to its possible implications. These two approaches are too new and exciting at this point to do more than give us a sense of anticipation that there is much more to come, and, in the framework of my title, that some of it might be practical.

Finally, we should not forget the host factor, because of our long tradition of considering the tumor as "the thing." That great numbers of cancer cells are carried through the veins draining a tumor for every metastasis that occurs (*25*), or that the ultimate success of radiotherapy, contrary to its immediate action, is improved by better nutrition (*26*), emphasizes the fact that the host should not be looked at as a simple culture medium for a clone of wild cells.

This has been more an essay that an exhaustive review. Time limitations have made that necessary. It can only be said that many new avenues have been opened up, that we have shed many of our older doctrines, that, in the tradition of this laboratory, cancer is becoming more and more a respectable aspect of biology, and that in the context of my title the prospects are at least somewhat brighter, though they look to be much more complicated, than ever before.

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U.S. GOVERNMENT PRINTING OFFICE: 1960

For sale by the Superintendent of Documents
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U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
Public Health Service • National Institutes of Health

NATIONAL CANCER INSTITUTE MONOGRAPH NO. 3

Conference on Experimental Clinical Cancer Chemotherapy

August 1960

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
Public Health Service

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NATIONAL CANCER INSTITUTE
Bethesda 14, Maryland

UNITED STATES OF AMERICA

Conference on
Experimental Clinical
Cancer Chemotherapy

Washington, D.C.,
November 11 and 12, 1959

Edited by
B. H. Morrison III

U.S. Department of Health, Education, and Welfare
Public Health Service • National Institutes of Health
National Cancer Institute

July 1960

National Cancer Institute Monograph No. 3

UNITED STATES
GOVERNMENT PRINTING OFFICE
WASHINGTON : 1960

For sale by the Superintendent of Documents, U.S. Government Printing Office
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The Future of the Cancer Chemotherapy National Program. Sidney Farber, *Children's Cancer Research Foundation, Boston, Massachusetts*

Indirect Benefits of the Cancer Chemotherapy Program.

Chemotherapeutic Agents of Confirmed or Potential Value in Human Cancer.

Introductory Remarks

DR. STUART SESSOMS, ***Chief, Cancer Chemotherapy National Service Center, Silver Spring, Maryland***

On behalf of the Cancer Chemotherapy National Service Center, its panels, committees, and staff, I bid you welcome to Washington and to this, the first of our clinical symposia. The Cancer Chemotherapy National Service Center was officially established in 1955. During the more than four years that have passed since that time, the clinical program, of which you are part, has grown to the magnitude of 500 clinical investigators representing approximately 200 hospitals.

This is the first time that we have had an opportunity to meet with all the clinical investigators and, as those of you who participated in the planning phase of this program are aware, there have been many differences of opinion as to how one could most effectively approach this type of meeting. We will be interested in obtaining your views with regard to the organization and conduct of this conference to guide us in future efforts of this kind.

The Cooperative Clinical Program

I. S. RAVDIN, ***University of Pennsylvania, Philadelphia, Pennsylvania***

ANYONE who has an adequate background in medicine or surgery must freely admit that once malignant disease has spread beyond the organ of its origin it is difficult to eradicate. We know that the time to cure cancer, except in the occasional instance, is when it is still strictly a local disease. With the therapy now available, early diagnosis and early adequate operation, or adequate radiation, for a limited group of cancers, are the only means at our disposal for significantly increasing the number of 5- and 10-year survivals. It is my conviction that further extension of surgical or radiation therapy alone will not greatly improve the presently accepted end results.

I say this with full realization that clinical cancer as we have come to know it is not a single disease and that the life history of even similar tumors in the same organ is often not the same. Internists, surgeons, and radiologists realize the inadequacies of existing therapy. Thoughtful clinicians admit freely that the present methods of treating a wide variety of spreading malignant lesions are unsatisfactory, if a high percentage of long survivals is to be expected. It is because of this that clinicians, internists, surgeons, and radiotherapists are attempting seriously to improve existing end results by other means of therapy.

In many of our universities and in certain of our distinguished cancer institutes, individuals are concerned with basic research in order that new knowledge may assist in unraveling the problems associated with the causes and nature of malignancy. In many of these institutions the same or other individuals are playing significant roles in the chemotherapy of late malignant disease, with the hope that through the use of these agents we may find better methods for the complete therapy of malignant tumors.

Cooperative effort is a frequent vehicle in university life. Closely coordinated efforts are more easily accepted in wartime, but are also achieved to a lesser degree in peacetime.

In many of these same institutions and in others, young surgeons are participating actively in the adjuvant chemotherapy program wherein

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[1] Presented at Conference on Experimental Clinical Cancer Chemotherapy, Washington, D.C., November 11, 1959.

chemical agents are being utilized at the time of, and immediately after, operation for major malignant lesions with the hope that certain of these agents will improve the end results now being attained by surgical means alone. There is some evidence suggesting that the results obtained by irradiation may be potentiated by the simultaneous administration of chemotherapeutic agents.

There are those who have stated that the adjuvant therapy program, as well as the chemotherapy program in general, is an irrational approach to the major problems of malignant diseases. They insist that until we understand the precise basic nature of cancer we cannot expect significantly to improve existing end results. I have patiently listened to what these individuals had to say, and I have patiently read what they have written, but I am not convinced by their argument. The history of other significant advances in therapy would suggest to me that perhaps those who believe that all progress will be made through basic science alone are not fully conversant with previous contributions to medicine, which over the years have come to affect significantly a variety of disorders.

Lister knew little about bacteriology when he developed the antiseptic theory for the control of infection at operation. Banting and Best made insulin available in 1921; 10 and 15 years later basic scientists were still working in order that we might have a clearer concept of the manner in which insulin acted. The sulfonamides began to be used in the mid-thirties, and 10 years later we were not sure of the biological processes by which these agents acted. Penicillin became available in 1943, and within the next 10 years an increasing number of the antibiotics were obtainable for clinical therapy, at a time when it was not entirely clear what the biological reaction was that resulted in the death of organisms controlled by these agents.

Those who oppose the chemotherapy program on the basis that it is in large part an irrational approach to the problem might well consider Paul Weiss' admonition, "Real science is earthly; just one of many branches of the human enterprise; one to be cultivated, but not a cult. Science must make more men its devotees, but none its priests." I might insist that the chemotherapy program is quite as rational an approach to the problem as is some of the basic research that is being done in the area of the malignant diseases.

It is said by some that surgeons ought not to be playing a significant role in the area of the chemotherapy of malignant disease. I must remind you of a statement by Harvey Cushing in his address on "Realignments of the Greater Medicine and Surgery" at the Third International Congress on Medicine and Surgery in June, 1913. He said, "The young surgeon of the future will be an internist first and a surgeon later."

The surgeons of this country really control at one time or another the majority of the solid tumors. Many are experienced investigators, and a worthwhile number have made significant contributions to our better understanding and solution of a variety of disorders. It is, moreover, a fortunate circumstance that internist and surgeon are working together in this important area, each functioning to the greatest extent in that particular area in which he has a major interest.

The idea that the extension of malignant diseases might be controlled by chemotherapy is not a new one. For some years, androgens and estrogens have retarded the spread of certain malignant tumors of the breast and caused regression in areas of metastases. Other agents have been helpful in certain of the leukemias. A variety of alkylating agents have been found useful in pulmonary cancer, and while none of these agents have been proved to cure a patient with widespread malignant disease, many of them have prolonged life. Sure it is that sufferers of late malignant disease have received better over-all therapy than ever before.

The data that are accumulating as a result of the active interest of competent clinical investigators have led to increased knowledge about the life history of many of these lesions. It is not illogical to consider that, while we are waiting to obtain a clearer understanding of the basic nature of cancer, surgery or irradiation, or both, and one or more of the chemical compounds now being used, or to be synthesized, may well implement present end results.

That a variety of malignant lesions have not responded with sufficient regularity to drugs is beside the point, for certain of the newly available compounds are showing some effect upon such previously unaffected tumors as the chorioepitheliomas. There is agreement that a number of university services participating in programs of cancer chemotherapy have clarified, to a degree, certain of the mechanisms of the disease, and have beyond question improved the therapeutic management of the cancer patient. Furthermore, they have provided objective criteria that can be used in the evaluation of antitumor drugs.

There are inherent difficulties in this program. It cannot be prophesied at the present time that antitumor drugs effective in available screens will, with any degree of accuracy, be active when used in man. We must admit that available screens are as yet not good enough.

It has been difficult to obtain a better understanding with the Chemotherapy Study Section. There have existed differences of opinion in the approach to the general problem. Perhaps a reorientation of the mechanism of choosing projects will be the solution.

It has been said, "The surgical cooperative chemotherapy program has been entirely concerned with the clinical evaluation of experimental evidence which indicated that (1) early spread of cancer cells can be curtailed by chemotherapy given shortly after tumor dissemination, and (2) therapeutic results with chemotherapy can be enhanced by decreasing the size of the tumor to be treated through partial surgical removal." I think I have lived with this program fairly carefully since its inception, and I cannot agree that the objectives as stated concern more than but a minor fraction of the activities of the surgical investigators participating in the cooperative program.

The cancer chemotherapy program of the National Cancer Institute was created to discover effective substances for the treatment of malignant disease. In the 4 years that the program has been operating, the largest screening facility in the country has been organized. We earnestly hope that better methods of screening agents will be found. Seventeen clinical study groups have been formed in 200 institutions. Existing chemotherapy programs have been implemented and new programs initiated. In addition, through increased grant support and a new mechanism for the support of research—the contract—hundreds of interrelated research projects are being carried on throughout the nation in universities, hospitals, government laboratories, and private industry.

We need seriously to extend the existing studies being carried out. The ovarian tumors, as well as the prostatic tumors, require further careful evaluation from the standpoint of chemotherapy.

It must be admitted that the program has contained some imbalances due to its rapid growth, yet, in spite of this, a sound basic structure has been built on a framework of cooperating scientists and organizations throughout this country.

As the chemotherapy program has developed, a number of indirect effects have become evident, the two most outstanding of which are (1) more scientists are studying the problems of cancer than would have in the normal course of events and (2) a more universal definition of the cancer problem and therapy has been made. Increased knowledge from the work done has further emphasized the need for additional studies on many biologic, biochemical, and biophysical processes occurring in both healthy and cancerous cells. There are additional indirect effects that have been realized through this program, for it has assisted in the training of hundreds of scientists and clinicians, not only in cancer research but in the basic sciences.

The program has gained the confidence and active collaboration of the pharmaceutical industry. It has stimulated the synthesis of unusual chemicals and the production of a wide variety of drugs of unusual types in quantities previously unavailable at all, or unavailable in amounts necessary for clinical trial. It has established large facilities for preclinical pharmacology and toxicity testing. It has made available new steroids for clinical trial, for assay, and for experimental tumor work. It has revived and stimulated interest in the physiological aspects of the tumor-host relationship through attempts to find tumors of endocrine significance, and in this way it has helped to broaden the scope of the attack on malignant disease.

The organized cooperative clinical studies have yielded solid quantitative data in a much shorter time than any single institution could possibly have achieved. The program has stimulated the interest and cooperation of many internists and surgeons in the care and chemotherapy of advanced malignant disease. It has organized a national standard reporting system of end results in malignant disease, and such reports are now being made on more than 50,000 new cancer cases a year. It has developed biometric units in a number of our medical schools and other institutions to provide statistical consultation not only for cancer chemotherapy but for other research. It has made available the first truly unselected, complete data, on a national basis, of operative complications and mortality from gastric, pulmonary, and rectal resection, and it has set the pattern for other research areas that also need extensive inter-institutional cooperative effort.

The gains have been substantial, and while we have not achieved our goal, neither have those who have been investigating the basic nature of cancer to the end that an approach, which they believe to be the more rational, will lead to the circumstance whereby better methods of therapy will be found. There is in this large field of disease an adequate place for all competent investigators; from both groups additional new knowledge is sure to come. We must, if we are to progress, understand that "understanding science is a way of thinking and science's task is to serve man."

The present critical evaluation of the entire cancer chemotherapy national program is nearing completion. The conclusions will bring together the results of critical study by scientists and physicians who compose the many panels and committees working through the Cancer Chemotherapy National Service Center under Dr. Sessoms. Certain generalizations may be made now.

To the best of our knowledge no scientist has been denied support for any project that suggests a rational approach to the chemotherapy of cancer. Through the Center, the work of chemists and biologists seeking rational approaches has been accelerated and facilitated to a degree that would have been impossible without a central organization.

The bulk of the chemicals examined during the past 2 years have come from the antibiotics programs of industry and consist of crude beers. No rational approach is possible in the selection of anticancer antibiotics. Skill and experience, however, will account for the greater success of some laboratories. This program must be largely empirical.

The rapid progress in the study of hormone analogues could not have been achieved by individual or uncoordinated effort.

A similar study of the vast number of plant natural products is being undertaken.

The three-tumor mouse screen is being subjected to careful analysis. This method, as well as all other screening methods, can be evaluated only when a sufficient number of candidate compounds show true effectiveness against tumors in man. The vast majority of scientists interested in cancer chemotherapy are prepared to drop any methods of screening presently employed when a more specific method is discovered. Until then it is essential to continue along the present lines.

Preclinical pharmacology must be expanded to meet the needs of the clinician. It is understandable that pharmacologists are not anxious to undertake huge programs of preclinical pharmacology without assurance that the compound is of real interest to clinicians. It is also clear that the clinician can evaluate the anticancer properties of a compound more quickly and accurately if he has precise information concerning time-dose relationships, excretion, metabolism, and distribution in the tumor and in the body.

The cooperative clinical studies carried out with the aid of the Center through its clinical panel have been responsible for achievements that could not have been brought about by any other organizational pattern or sponsorship.

The large number of institutions and doctors who have voluntarily agreed to study the response to anticancer agents are organized to work together in small groups on the basis of protocols representative of specific experimental designs adopted as representative of the best methods of evaluation of chemotherapeutic agents.

Extensive modifications may arise from the critical evaluations presently under way. The greatest importance of the clinical cooperative trials is that a large scale attempt has been effectively carried out for actual clinical investigation based upon predetermined criteria. Data emanating from such studies permit analysis that otherwise could not be possible. The goals of individual studies in this cooperative plan were selected as much to achieve familiarity with this kind of clinical investigation as for the clinical investigation itself. It must be remembered that this kind of work is not as exciting as research of an individual scientist. It calls for obedience to rules, democratically achieved and agreed to. A by-product of such clinical interest is the great improvement in the care of patients with advanced cancer, and the conveyance of hope to the patient because of the increased tempo of research activity concerning his problem. An important by-product, too, is the collection of information concerning the life history and biologic behavior of the various forms of cancer. These by-products alone are worth the effort of the entire clinical program so far.

CONCLUSION

Careful and severely critical evaluation of all aspects of the cancer chemotherapy program should be made constantly as the program advances, with provision for periodic analyses of more formal nature. Criticisms of the program should be made on the basis of a knowledge of its purposes and the administrative structure erected to achieve them and also on the basis of our deficiencies in knowledge in the various aspects of the cancer problem. There is often no dividing line worthy of mention between basic and applied research in the chemotherapy of cancer. While the search for exploitable differences between the normal and cancer cell continues, the controlled and guided empirical program may achieve a satisfactory solution, after which we can attempt to determine how the effect of a chemical works. We are not confronted by a choice between basic and applied research. Those responsible for choosing the directions of research must and do recognize that receptiveness of new ideas must go along with vigorous and realistic development of ideas that currently appear to be of greatest promise.

Only after the goal has been reached will one be able to look back and state without fear of dispute which one of the many roads was the one we should have taken in the first place.

The adjuvant program is based upon clinical and biologic information concerning the nature and behavior of cancer. It is a logical approach and demands immediate and long-term study by groups working in cooperation according to a preselected design.

Finally, the increasing number of potential anticancer agents necessitates as rapid an increase in the number of clinical investigation groups as we can make. The two hundred studies presently in the clinical program represent only one part of the total strength in clinical cancer research. This decision should be made final only after the present evaluation study has been completed. The bottleneck now resides in the ability of clinical groups to conduct rapid, critical, and effective evaluation of the anticancer agents available.

Chemical Structure as Related to Antitumor Action [1]

BERNARD R. BAKER, *Department of Biological Sciences, Stanford Research Institute, Menlo Park, California*

Many reviews have been written on the antimetabolite theory, such as that of Woolley (*1*), and on chemical agents that have selective effects on mammalian tumors (*2*, *3*). The first type of review has considered only competitive enzyme inhibitors, while the second type usually has not differentiated between competitive and irreversible enzyme inhibitors. I shall attempt to defend the thesis that most, if not all, anticancer agents are effective because of this irreversible nature. In this defense, I shall not try to quote all the pertinent references, nor shall I try to separate fact from fancy. My main intent is to encourage thought that may stimulate more experimentation to separate fact from fancy in this nearly virgin field.

As a foundation for this talk, I should like to define my usage of the terms *competitive*, *noncompetitive*, and *irreversible enzyme inhibitors* as well as *competitive* and *noncompetitive reversing agents*, since these terms have different meanings to different investigators. Consider the following two enzymatic reactions, where M is a metabolite (or substrate) and F_1X is a cofactor, F_1 bearing a group to be transferred (X), and F_2Y is a second cofactor, F_2 bearing a group to be transferred (Y).

$$M + F_1X \xrightarrow{\text{enzyme 1}} MX + F_1$$

$$MX + F_2Y \xrightarrow{\text{enzyme 2}} MXY + F_2$$

This sequence represents part of the anabolic building of essential cellular material. When the arrows are written in the opposite direction, the process represents the catabolic breakdown of a no longer used cellular material.

If an antagonist, M, of the metabolite M is added to this system, the rate of the enzymatic reaction for formation of MX will be slowed. If the percent decrease in rate remains the same over a wide concentration of M when the ratio of $\frac{M'}{M}$ is held constant, then M′ is considered a competitive inhibitor. If the percent decrease in rate is not constant when the ratio of $\frac{M}{M'}$ is held constant, then M′ is a noncompetitive antagonist. In contrast, M′ is an irreversible inhibitor when no generation of MX takes place, regardless of the concentration of M and F_1X, providing sufficient M′ has been added to saturate the enzyme.

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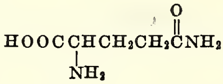
[1] Presented at Conference on Experimental Clinical Cancer Chemotherapy, Washington, D.C., November 11, 1959.

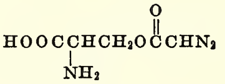
When M′ is a competitive antagonist, it is obvious that M is a competitive reversing agent. Defining a noncompetitive reversing agent requires considering 2 coupled enzymes. Adding any of 3 types of M′ to this coupled enzyme system can have no effect on the generation of MXY if sufficient MX is added to the system. Thus MX is a noncompetitive reversing agent, sometimes called an end-product reversing agent.

There are two known types of irreversible inhibitors. The first type fits the enzyme site for M, then combines chemically with the apoenzyme or coenzyme. The formation of a covalent bond stops the desorption process of M′; thus no M can get on its enzyme site to be converted to MX. For all practical purposes the enzyme has become deactivated, though selectively. Similarly, a cofactor inhibitor, F′X or FX′, can be irreversible. The glutamine antagonists L-azaserine (II) and 6-diazo-5-oxo-L-norleucine (DON) (III) are presumably of this type. The enzyme that converts formylglycinamide ribotide to the corresponding amidine, with glutamine (I) as a cofactor, is rapidly and irreversibly inactivated when either L-azaserine or DON is added to the system (*4*). Other glutamine transamidases are inactivated similarly. However, enzymes that do not use glutamine, *e.g.*, serum albumin (*5*) and glutamic dehydrogenase,[2] are unaffected by L-azaserine and DON. Thus, L-azaserine and DON are specific irreversible inhibitors for glutamine transamidases and are not general protein denaturants.



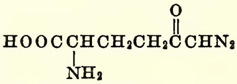
$$HOOC\underset{NH_2}{CH}CH_2CH_2\overset{\overset{O}{\|}}{C}NH_2$$

I



$$HOOC\underset{NH_2}{CH}CH_2O\overset{\overset{O}{\|}}{C}CHN_2$$

II



$$HOOC\underset{NH_2}{CH}CH_2CH_2\overset{\overset{O}{\|}}{C}CHN_2$$

III

The second type of irreversible inhibitor also fits the enzyme site for M, but is held so strongly to the enzyme by the electrostatic bonds that M cannot displace the inhibitor from the enzyme surface at any reasonable physiological concentration. Again, for all practical purposes, the enzyme has become selectively deactivated. A number of 2,4-diaminopyrimidines and closely related structures appears to inhibit folic reductase in this manner.

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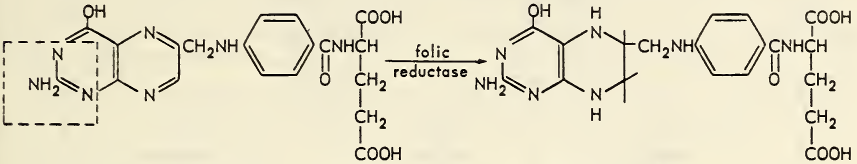
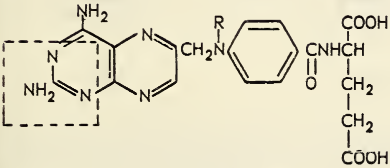
[2] Lee, W. W., and Baker, B. R.: Unpublished results.

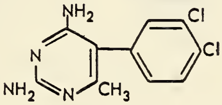
Folic acid (IV) is converted to its cofactor form, tetrahydrofolic acid (V), by the enzyme folic reductase, TPNH being required for the reduction. Antagonists of folic acid (IV) such as aminopterin (VI), amethopterin (VII), the Daraprim analogue (VIII), and the triazine (IX) inhibit this enzyme system irreversibly. Evidence that aminopterin (VI) and amethopterin (VII) are type II irreversible inhibitors was recently shown by removing the aminopterin by extended dialysis against folic acid, which thus regenerated the enzyme (*6*); these antagonists were bound 10^5 times stronger than folic acid. Probably folic reductase that has been inactivated by VIII and IX could also be reactivated by dialysis.



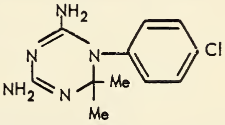
IV V



VI, R = H
VII, R = Me



VIII



IX

The effect of VI to IX as type II irreversible inhibitors can be rationalized if it is assumed that the 2-amino group (shown in box) of folic acid is one of the binding sites on the substrate attached to the enzyme. Since this site is basic, the corresponding enzyme site must be acidic. When the 4-hydroxyl of folic acid (IV) is changed to an amino, as in aminopterin (VI), then the 2-amino group becomes a much stronger base, resulting in a stronger electrostatic attraction between the 2-amino group and the acidic site on the enzyme. This stronger attraction could be sufficient to stop desorption of VI from the enzyme, and would thus deactivate the enzyme.[3]

The interesting anticancer agent 5-fluorouracil (X) is converted to the active form, the deoxyribotide (XI), in the cell (*7*). The latter is an irreversible inhibitor of thymidylate synthetase (*8*), the enzyme that converts deoxyuridylic acid (XII) to thymidylic acid (XIII) in the presence of tetrahydrofolic acid (V) and formaldehyde. 5-Fluorouracil (X) and its nucleosides are known to be much more strongly acidic than uracil and its corresponding nucleosides. If the acidic portion of XI and XII shown in the box is assumed to be a binding site on the enzyme, then the

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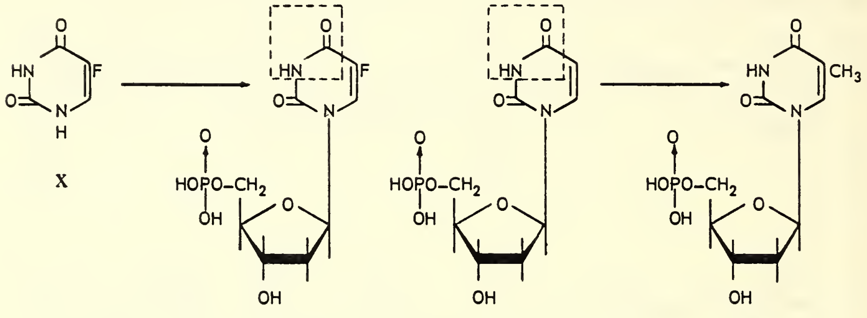
[3] Even though absorption of aminopterin is reversible from the kinetic point of view, the presence of equal amounts of aminopterin and folic acid in a cell will slow the reduction of folic acid by a factor of 10^5. Thus from the biological standpoint, the effect by aminopterin would be that of an irreversible inhibitor, that is, no significant amount of tetrahydrofolic acid (V) would be synthesized.

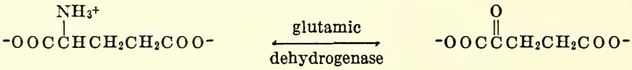
NH of XI, being much more acidic than the NH of XII, would cause XI to bind more strongly to the enzyme than XII. If this binding is sufficiently strong, then XI is not desorbed and is a type II irreversible inhibitor. It is less likely that XI is a type I inhibitor. The fluorine atom is probably not active enough to be replaced to form a covalent bond with the enzyme.



X XI XII XIII

Among the most important considerations in designing antimetabolites are the binding sites of the metabolite. If the potential inhibitor cannot bind to the enzyme, it obviously cannot be an inhibitor. Examples of usual anionic binding groups are phosphates, carboxylates, acidic amides, phenolic hydroxyls, and mercaptans. Examples of cationic binding groups are amines of various types and sulfonium ions. Relatively neutral binding groups are alcohols, ethers, aryl rings (*9*), sulfides, neutral amides, and halogens; these usually exhibit poor binding of a secondary nature, and when only such a group is present, the Michaelis constant for binding is poor.

It is possible to determine experimentally the binding sites of a substrate by competitive inhibition measurements with various potential inhibitors. For example, Hellerman *et al.* (*10*) investigated a variety of compounds for their effect on glutamic dehydrogenase. Whereas glutarate (XVI) is an excellent inhibitor of glutamate dehydrogenase, glutamine—even though it has nearly the same dimensions as glutamate (XIV)—showed no appreciable inhibition of the enzyme reaction. Thus, both carboxylates of XIV are binding sites and the NH_3^+ is not necessary for binding. If the NH_3^+ and α-COOH of

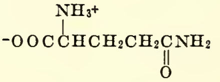


$$^{-}OOC\overset{\overset{NH_3^+}{|}}{C}HCH_2CH_2COO^{-} \xleftrightarrow[\text{dehydrogenase}]{\text{glutamic}} {}^{-}OOC\overset{\overset{O}{\|}}{C}CH_2CH_2COO^{-}$$

XIV XV

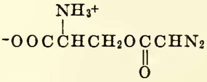
$^{-}OOCCH_2CH_2CH_2COO^{-}$

XVI



$$^{-}OOC\overset{\overset{NH_3^+}{|}}{C}HCH_2CH_2\underset{\underset{O}{\|}}{C}NH_2$$

XVII



$$^{-}OOC\overset{\overset{NH_3^+}{|}}{C}HCH_2O\underset{\underset{O}{\|}}{C}CHN_2$$

XVIII

glutamate (XIV) were the binding sites, then glutamine (XVII) should have been effective and glutarate ineffective.

If one did not consider binding sites, but only general antimetabolite theory (*1*), one might suspect that azaserine (XVIII) could inhibit glutamic dehydrogenase, since only the —CHN_2 group has been substituted for the OH group in glutamate (XIV), a small change. However, when binding sites are considered, it would be surprising if azaserine had any effect on glutamic dehydrogenase. That azaserine has no inhibitory effect on glutamic dehydrogenase has been verified experimentally (*see* footnote 2).

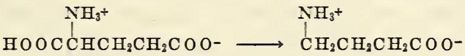
It also follows that glutamine, as well as its inhibitors, azaserine and DON, have α-COOH and NH_3^+ binding when bound to enzymes that are glutamine transamidases.

These observations suggest a useful rule of thumb for predicting some polar groups that cannot be binding sites. Consider the original general expression for an enzymatic reaction:

$$MX + F \rightleftarrows FX + M$$

The rule of thumb would state that X cannot be a binding group, nor can the functional group on M or F where X must be accepted be a binding site. Thus the NH_3^+ group of glutamate (XIV) and the oxo group of α-ketoglutarate (XV) are not binding sites in the glutamic dehydrogenase system. Similarly, when glutamine transfers its amide NH_2 group, the carboxamide function of glutamine cannot be a binding site on the enzyme.

It can be predicted that neither the carboxamide of glutamine nor the γ-COOH of glutamic acid are binding sites on the enzyme that interconverts glutamine and glutamate. Similarly, it can be predicted that the α-carboxyl group of glutamate is not a binding site on the enzyme that decarboxylates glutamate (XIX) to γ-aminobutyric acid (XX). These examples



$$HOOC\overset{NH_3^+}{\overset{|}{C}}HCH_2CH_2COO^- \longrightarrow \overset{NH_3^+}{\overset{|}{C}}H_2CH_2CH_2COO^-$$

XIX XX

serve to illustrate the different types of binding that can take place in a polyfunctional metabolite such as glutamic acid.

The second important consideration in designing an antimetabolite is the placement of the fraudulent group. Let us now consider the binding of type II irreversible inhibitors, *i.e.*, those inhibitors that bind irreversibly by electrostatic attraction to the enzyme. Introducing the fluorine atom, as used in 5-fluorouracil, is an excellent example of a change to increase acidity. The fluorine atom is small and should cause no steric hindrance to enzyme binding. Thus, if a fluorine atom can be introduced next to an acidic binding group, stronger binding with the hoped-for lack of desorption and resultant irreversible inhibition may occur. But beware introducing a fluorine atom adjacent to a basic binding group! This change will cause a lessening of basicity with resultant loss of binding and the compound will show little inhibition.

The increase in basicity resulting from replacing a hydroxyl with amine, as with the inhibitors of folic reductase, has much less apparent utility.

It seems to me that the type I irreversible inhibitor has much more general use. There are many possible variants of this type of inhibitor that operate by fitting the enzyme site for a substrate and then alkylating the enzyme system.

Two classes of type I inhibitors can be envisioned. The first class requires a small, but specific, change in the substrate at or near the group X in FX or MX; these are classified as "endo-alkylating" inhibitors. Let us consider azaserine and glutamine transamidases in more detail.

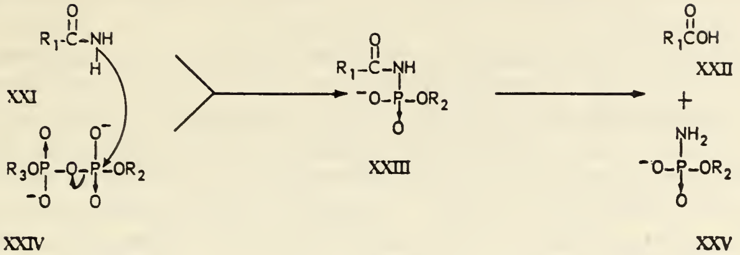
All glutamine transamidases seem to require ATP. Most likely the ATP is required for activating the carboxamide group so that the NH_2 group can be transferred (*11*), and possibly also for activating the NH_2 receptor group. Several possible mechanisms can be written for activating the carboxamide group. In scheme 1, a pyrophosphate (XXIV) such as ATP reacts with an amide (XXI) such as glutamine to form the imide (XXIII). The hydrolytic lability of the imide (XXIII) should allow ready hydrolysis to the phosphoramide (XXV), which can be the active aminating agent. Cossano and Sankar (*12*) have recently shown that an enzyme from rat liver can cause ATP and NH_3 to react to form AMP-NH_2, which in turn, is the active form of ammonia for the enzymatic transamination of such keto acids as α-ketoglutarate. Thus it is possible that AMP-NH_2 is also the active aminating form derived from glutamine.

In scheme 2, the amide (XXI) can be activated by reaction of the pyrophosphate (XXIV) to form an imino ester (XXVI); the latter should be an active aminating agent (*11*).

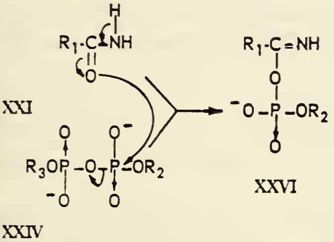
No matter what the mechanism for activating the carboxamide of glutamine with ATP may be, it is clear that ATP and glutamine must be held in juxtaposition on the enzyme surface so that an essentially unimolecular reaction can take place to activate the carboxamide group, as indicated in schemes 1 or 2. Since azaserine (XVIII) or DON (XVII) can be held by the enzyme in place of the glutamine, as shown in scheme 3, the diazoketone group of XXVII is in position to alkylate one of the phosphate groups to form XXVIII, which thus irreversibly binds the azaserine or DON (*11*). Of course, the diazomethyl group could also simply alkylate some active group such as NH or OH on the enzyme site.

This principle of "endo-alkylation" should have considerable utility. Any substrate bearing a carboxyl or carboxamide that must be activated by ATP for accepting a group can possibly be converted to an irreversible inhibitor by transforming the carboxyl or carboxamide group to a diazoketone. Whether other small alkylating groups, such as $FCH_2\underset{|}{C}=O$, can be substituted for a carboxyl group to obtain inhibitory properties also remains to be determined.

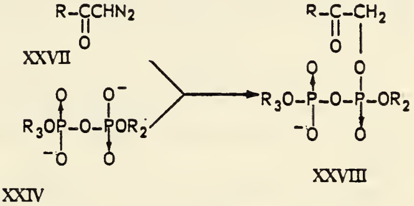
SCHEME 1



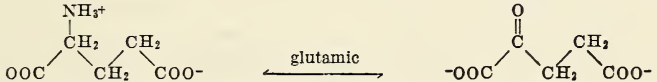
SCHEME 2



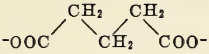
SCHEME 3



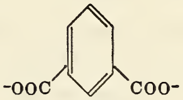
A second class of type I inhibitor, one which combines irreversibly with the enzyme by "exo-alkylation," is theoretically possible, but no specific examples have yet been established. To develop this thesis for "exo-alkylating" inhibitors, it is necessary to return to some examples of competitive inhibitors so that further light may be shed on some of the critical dimensions of an antimetabolite.



XXIX XXX



XXXI



XXXII

Caughey *et al.* (*10*) observed that both glutarate (XXXI) and isophthalate (XXXII) effectively inhibited glutamic dehydrogenase to the same extent, but that phthalate and terephthalate were much less effective. Although glutamate (XXIX) can assume a number of different conformations, isophthalate (XXXII) is rigid. Since isophthalate fits the enzyme site as well as glutamate (*10*), it is clear that glutamate must assume the conformation equivalent to isophthalate when attached to the enzyme, as shown by the drawing of XXIX. Hellerman (in *10*) concluded, ". . . it is apparent that the portion of the molecule oriented toward the enzyme surface is structurally much the same in both these compounds." It is also obvious that the remainder of the benzene ring constitutes a considerable change in gross measurements compared to glutamate (*13*).

Inhibitors of the enzymatic oxidation of succinate to furmarate can vary considerably in gross measurements, provided the change is only at one α-position of the succinate. Thus α-alkylsuccinic acids, even with large alkyl groups, competitively inhibit succinic dehydrogenase (*14*).

Phenoxyacetic acid has been observed to be a competitive inhibitor of lactic dehydrogenase (*15*). A number of substituted phenoxyacetic and β-phenoxypropionic acids inhibit lactic dehydrogenase even more effectively than the parent phenoxyacetic acid.[4] Bulk on the aryl ring caused little or no effect on the I_{50},[5] whereas substitution of chlorine on the ring made these compounds more effective inhibitors. For example, phenoxyacetic acid has $I_{50} = 3.0$; 2,4-dichlorophenoxy acetic acid has $I_{50} = 1.6$.

The large variation in size and position of substituents on the phenoxy ring, which can be made without losing inhibitory activity, shows that the phenyl ring is on the side of the inhibitor that is away from the enzyme surface.

Although Woolley (*1*, p. 227) has stated that an antimetabolite should be as close as possible in structure to the corresponding metabolite, his concept should be modified to state that an antimetabolite should be as close as possible in structure to that part of the molecule where the stereospecific requirements of the enzyme surface must be met (*13*). The examples of inhibitors cited for the three enzymes systems certainly substantiate the fact that there can be space on the metabolite that is away from the enzyme surface where considerable change in size can be made without influencing adsorption on the enzyme surface. The amount and optimum position of space available will certainly vary with each enzyme; these parameters will have to be determined on each enzyme individually by the study of potential inhibitors in the manner described for phenoxyacetic acid inhibition of lactic dehydrogenase.

If the working hypothesis is now acceptable that there is room for substitution on the back side of a metabolite, we can return to the design of an irreversible inhibitor that could theoretically operate by "exo-alkylation" of the enzyme system. Phenoxyacetic acid as an inhibitor of lactic dehydrogenase makes a suitable model to explain this type of irreversible inhibitor. It has been established in this case that the phenyl ring is away from the enzyme surface when phenoxyacetic acid fits the site for lactic dehydrogenase. On the enzyme surface, near the site of attachment, are active hydrogens derived from such functional groups as amino, carboxyl, carboxamide, hydroxyl, and mercapto, and which can be replaced by alkylation. If a suitable alkylating group is placed on the phenyl ring of phenoxyacetic acid in such a position that the alkylating group can bridge to a group bearing an active hydrogen and alkylate at that site, then a covalent bond will be formed; the formation of this bond obviously makes the inhibitor irreversible. Which alkylating group should be used, what position it should have on the phenyl ring, and what distance the alkylating group must be from the phenyl ring are points that can be answered at this time only by synthesizing and evaluating suitably substituted phenoxyacetic acids (*see* footnote 3). Among the groups that should be evaluated are diazoketones, diazonium salts, carboxazides, halomethyl ketones, epoxides, ethylenimines, mono-β(chloroethyl)amines, and bis-(β-chloroethyl)amines. The last three fall into the class of nitrogen mustards. It is possible that some nitrogen mustards attached to a substrate as a carrier group operate by the carrier fitting the site for the substrate, with the alkylating group combining irreversibly with some nearby active site. Thus this type of irreversible inhibitor should also be enzyme specific.

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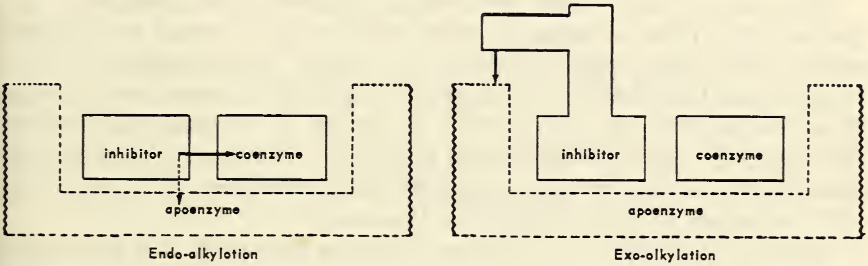
[4] Skinner, W. A., Martinez, A., and Baker, B. R.: Unpublished results.

[5] Millimolar concentration required to give 50 percent inhibition in the presence of 1 millimolar concentration of substrate.



TEXT-FIGURE 1.—Oversimplified diagram of endo- and exo-alkylation. *Arrows* represent the attack of an alkylating group on an active site. The two different routes for endo-alkylation are shown by the *solid arrow* and by the *dotted arrow*.

If this concept of irreversible combination of a metabolite bearing a properly orientated alkylating group with a suitable site can be reduced to practice with some authentic examples, the concept should have considerable application in the field of chemotherapy in general and cancer chemotherapy in particular.

A simplified diagram of endo- and exo-alkylation is shown in text-figure 1.

To this point we have only considered how one might design an irreversible inhibitor that can be fairly specific for a given enzyme. It is time now to consider what specific enzymes would be blocked to give inhibition of tumor growth with minimal effects on the host. Unfortunately, there is no simple answer to this problem, but enough basic information exists to suggest that certain areas of metabolism are more important than others.

Earlier antimetabolite theory (*1*) suggested that it was necessary to block an enzyme system present in the invading cell but not present in host cells. The sulfa drugs represent the classic example that led to the original Woods-Fildes concept of antimetabolites. Subsequent work did show that susceptible bacteria needed *p*-aminobenzoic acid for synthesizing the essential cofactor, tetrahydrofolic acid, a sequence blocked by sulfanilamide. It was also established that mammalian cells used a different route, reduction of folic acid.

Lengthy searches among the enzymes present in cancer cells and normal cells failed to find an enzyme present in a cancer cell that was not present in normal cells (*16*). Recently, quite the reverse has been observed, *i.e.*, a number of enzymes were absent from cancer cells that were present in normal cells. These results led Potter (*17*) to propose the "deletion hypothesis," which provides a useful baseline for selective antimetabolite action against tumor cells.

Curiously enough, the enzymes so far discovered to be absent in cancer cells are all catabolic, particularly those enzymes which degrade purines, pyrimidines, and aromatic amino acids. These areas of metabolism are, therefore, the ones now available where exploitable biochemical differences exist between normal and neoplastic tissues.

How, then, should these biochemical differences be exploited? If a purine, pyrimidine, or aromatic amino acid can be converted to a properly substituted enzyme inhibitor, then the normal tissue should be able to degrade and thus detoxify the inhibitor; susceptible tumor tissue, lacking these degradative enzymes, would be unable to degrade the inhibitor and would, in effect, be selectively poisoned. That some drugs do show selectivity of action by this mechanism has been shown by some of Potter's colleagues at the University of Wisconsin.

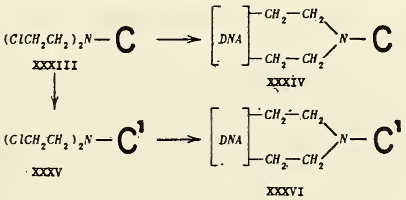
Harbers *et al.* (*18*) have demonstrated convincingly that 5-fluorouracil is degraded in normal tissue to α-fluoro-β-alanine, whereas susceptible cancer tissue degrades 5-fluorouracil much more slowly. In fact, there is probably a correlation between the effectiveness of this agent on a given tumor and the tumor's lack of ability to degrade the agent.

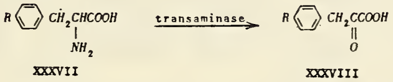
Similarly, Moore and LePage (*19*) have observed that thioguanine is degraded by nonsusceptible normal tissue to thiouric acid, a nontoxic compound. In contrast, susceptible cancer tissue degrades thioguanine to thiouric acid much less efficiently. Probably, 6-mercaptopurine also operates by selective oxidation.

Few anticancer agents have yet been studied in sufficient detail to state whether they act as irreversible inhibitors or competitive inhibitors. Certainly, aminopterin and related folic reductase inhibitors, 5-fluorouracil, L-azaserine, and 6-diazo-5-oxo-L-norleucine, are irreversible inhibitors. To my knowledge, there is no known anticancer agent that has been unequivocally proved to operate as a competitive antagonist. Even if such a competitive anticancer agent should arise, the field of irreversible inhibitors still has a strong position. In fact, these irreversible inhibitors were discovered by serendipity as a result of small changes in the corresponding metabolite. Another strong argument in favor of irreversible inhibitors is that much lower concentrations will be needed, since one inhibitor combines with one active site on an enzyme. In contrast, competitive inhibitors must compete with substrates and one enzyme molecule can convert many substrate molecules; if these substrates are present in high concentration, only an irreversible inhibitor that reacts with the enzyme will be able to block utilization of the substrate *in vivo.*

The two essential features necessary for a candidate drug to operate by the detoxification mechanism should also be applicable to the field of alkylating agents. The latest information available indicates that nitrogen mustards as a class of compounds react with DNA as the primary site of action; since this is an irreversible reaction by alkylation of DNA, it falls into the scope of this paper. If the nitrogen mustards are considered as a class of compounds bearing a carrier group, as represented by structure XXXIII, then the reaction product with DNA would have structure XXXIV.

It is also becoming clear that the carrier group can cause a change in cytotoxic concentration, that is, the same nitrogen mustard group attached to two different carriers may vary 20-fold in toxicity to the host. If such is the case, then the deletion hypothesis could be used to design better alkylating agents.





Let us consider phenylalanine mustard (Sarcolysin) as a hypothetical example. This compound can be represented by structure XXXIII where C is phenylalanine, or by structure XXXVII where R is $(ClCH_2CH_2)_2N^-$. It is well established that a number of tumors have a deletion of phenylalanine transaminase, the enzyme that converts phenylalanine (XXXVII, R = H) to phenylpyruvic acid (XXXVIII, R = H). As I have discussed earlier, many enzymes have room to allow a considerable structural change on the back side of a substrate and still utilize the modified substrate. Thus, phenylalanine mustard (XXXVII, R = $(ClCH_2CH_2)_2N^-$) might still be accommodated by phenylalanine transaminase and be converted to phenylpyruvic acid mustard (XXXVIII, R = $(ClCH_2CH_2)_2N^-$). If phenylpyruvic acid mustard is less toxic to the host than phenylalanine mustard, then the latter will show a selective toxicity against a tumor whose phenylalanine transaminase has been deleted.

Stated in another way, the carrier group C (XXXIII) can be converted to a less toxic mustard with carrier group C′ (XXXV), a reaction that the tumor cell with the deleted enzyme cannot carry out.

Although hundreds of alkylating agents have been synthesized and tested, only a few have been made with carriers that are normal substrates subject to metabolic change. Thus, the deletion hypothesis may serve to suggest additional alkylating agents to be made that may have a specific effect on a given tumor, depending upon the enzyme(s) deleted when the normal cell became a tumor cell. On this basis, it is my opinion that the field of alkylating agents is just at the frontier, rather than being a field that is well mapped and obsolete.

Few researchers have deliberately attempted to design and synthesize an irreversible inhibitor and few have looked at mechanism of action from this standpoint. My main intent has been to encourage thought that may stimulate investigators to more experimentation in this nearly virgin field of irreversible inhibitors, for in this relatively unexplored field may lie the answer to cancer chemotherapy.

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Discussion

Dr. Baker (Boston): Is this deletion phenomenon present in embryonic tissue and rapidly growing tissue, such as the hematopoietic system?

Dr. Bernard R. Baker: This phenomenon is present in regenerating liver and in the fetus. One of the problems being studied in cancer chemotherapy is: Why does liver stop regenerating when it reaches its normal size, and what is the control mechanism? This I would classify as a nutritional approach, since some factor is missing which must be added back. This is an excellent approach, I think. Those tissues with the same enzymes deleted, including stomach epithelial cells and bone marrow, are fast-growing tissues in which we see toxicity. For example, bone marrow is low on the enzyme xanthine oxidase. Since these cells cannot convert thioguanine to thiouric acid, one sees toxicity to these cells. I have been careful not to say all normal tissues. Certainly there is a hierarchy of susceptibility of normal tissues; that is, not all normal cells have the same enzyme capacity.

Dr. Burchenal (Sloan-Kettering Institute): How much would this deletion hypothesis help if the most sensitive normal cells also lack this enzyme so that there would be the same reaction on them as on the tumor?

Dr. Bernard R. Baker: Dr. Burchenal asks how the deletion hypothesis can work if a vital system such as bone marrow is also affected. Dr. LePage at Stanford Research Institute is making some progress to answer this question. Thioguanine and azaserine are synergistic against certain tumors. The first side effect of thioguanine is on bone marrow and the first side effect of azaserine is on stomach linings. Dr. LePage has shown in animals that a half ratio of thioguanine and a half ratio of azaserine still give a full impact on the tumor cell, but one has cut the effect on the two most susceptible tissues to a half. Dr. Curreri's group at Wisconsin is now studying this combination clinically. I have not heard the results.

The deletion hypothesis certainly is no panacea in that it is going to solve all problems in cancer chemotherapy. It gives the chemists and the biochemists a useful working hypothesis; this is the first biochemical difference we have seen that is exploitable.

Question: You mentioned that there was a 20-fold toxicity in the various mustards and this is exciting. I think we can expect great things in the session on alkylating agents if you are referring to toxicity in man as compared with effects on tumors, but if the reference to toxicity is in some other context, will you comment on this.

Dr. Bernard R. Baker: What I am talking about is gross toxicity to the host; I am not even talking about tumor. It should be possible for normal tissues to convert an alkylating agent to a less toxic (by $\frac{1}{20}$) alkylating agent, whereas susceptible tumor tissue cannot. This is the working hypothesis on which we are trying to build some new alkylating agents. There have been about 700 alkylating agents made by now and evaluated. Many were easy to synthesize; now we are getting into the tough work where one should use a metabolite carrier which may be selectively metabolized in tumor versus normal tissue. This is why I expect to see a resurgence in the field of irreversible inhibitors in general and alkylating agents in particular.

Biological Screening Systems for Determination of Antitumor Activity [1]

C. CHESTER STOCK, *Sloan-Kettering Institute for Cancer Research, New York, New York*

THE letter inviting me to the conference also contained a lightly veiled suggestion that we talk about new things. This was rather disconcerting, and it took me about a month to make up my mind that I would come. I will try to say a few things differently. It may be that I will omit some points that should be mentioned, but I hope to stress a few things that need to be designated and also to indicate some ideas that have emerged as a result of current studies.

Those of us who are in the screening area and other related areas recognize our role as middlemen. In this spot, we are both dependent on, and have obligations to, the chemists and clinicians who have been so ably represented by our two preceding speakers, Dr. Ravdin and Dr. Baker. We must provide information that will be useful to these chemists and clinicians in order to conserve their valuable time.

For the clinicians we must select, as reliably as possible, new agents for their consideration and we must also document, as well as we can, any possible superiority of new variants of the older active agents. In turn, we are dependent on the clinicians for adequate testing of the materials that we select. If the compounds are tested, we expect to be given as much supplemental information as possible to show us why the compounds have been successful or have failed. It must be recognized that it is a long time—unless we are extremely fortunate, a minimum of 3 to 5 years—from the time of development of a new test object or conception of a new technique through obtaining the baseline information about that technique or test object to the time the test object has adequately demonstrated its uniquely useful activity, *i.e.*, by testing the materials that come through the screen, the preclinical pharmacology, and, of course, the clinical trial.

We must provide the chemists with reliable information as promptly and as quantitatively as possible so that their efforts may be guided in the synthesis of new compounds or in the fractionation and isolation of active materials from promising natural sources. We must look to the chemists for new products to test. Some of these will simply be additional compounds, some compounds that have been produced with imagination as well as enthusiasm, and some variants on an old theme, such as Dr. Baker indicated with respect to the alkylating agents. And these will be carefully considered variations. There is also the large area of fractionations of natural products.

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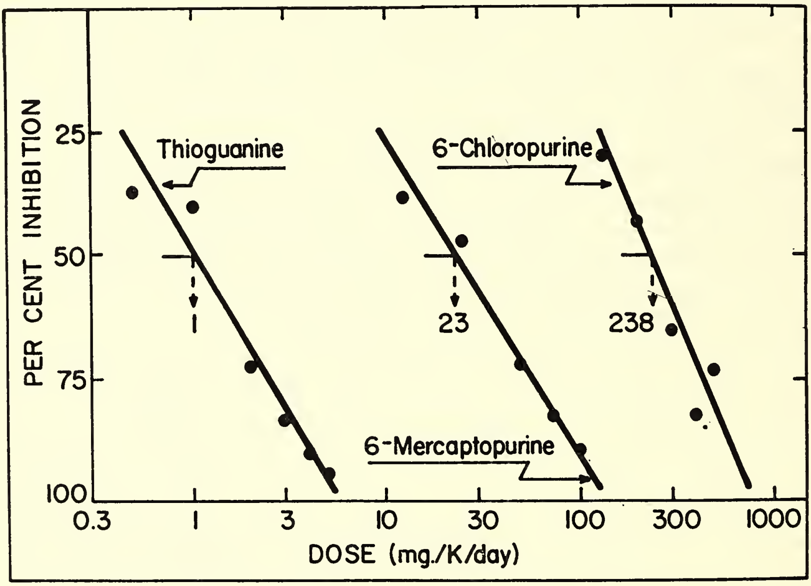
[1] Presented at Conference on Experimental Clinical Cancer Chemotherapy, Washington, D.C., November 11, 1959.

It is possible with some experimental tumors to obtain quantitative data (*1*). Text-figure 1 shows the dose-response curves with Sarcoma 180. With Adenocarcinoma 755 one can obtain even greater spreads from the minimal to maximal activity and thereby derive a fairly respectable therapeutic index, at least in the area of the purines. With Sarcoma 180 this has not been quite so easy; for example, the best of the materials effective against Sarcoma 180 have given what we might term a therapeutic index of no more than 3 to 8 (*2*, *3*), whereas with Adenocarcinoma 755 much greater indexes can be obtained. Dr. Reilly, in our laboratory, is trying to improve this situation where we have leads with respect to Sarcoma 180 (*4*).



TEXT-FIGURE 1.—Dose-response curves of Sarcoma 180.

I think most of us have had distinct reservations about the utility of the ascites form of different tumors. Recently, the Center experience has indicated that this is a very susceptible form of cancer, which gives too many leads that are false positives. Table 1 shows a few of Dr. Sugiura's data, which indicate that the solid form, in general, is more sensitive than the ascites form of a tumor (*5*), and that the material given intraperitoneally is usually much more effective; the test object is affected more by the intimate mixture with the injected dose. That this is not always true is demonstrated with the use of cortisone (table 1). The ascites tumor can possibly have a limited utility. I mentioned that Dr. Reilly is trying to develop an ascites test with Sarcoma 180, which may give us a much greater therapeutic index that will be useful to follow up the leads that have been obtained, especially with the natural materials with Sarcoma 180. This will enable us to give the chemist much better quantitative information to guide his fractionation.

Table 1.—Ehrlich carcinoma results

	Solid		Ascites	
Compound	SC	IP	SC	IP
HN2	±	±	++	++
TEM	±	±	±	++
8-Azag.	−	−	−	±
Cortisone	±	−	++	±

What about our test objects? Table 2 presents data, anonymous with respect to the compounds and the various tumors, which show that the experimental tumors respond differently to different agents (*6*). This is one of the reasons the Center adopted a three-tumor system for its primary screen—L1210 leukemia, Adenocarcinoma 755, and Sarcoma 180 (*7*). These tumors were selected on the basis of information of this type, and also because they had shown a limited but definite utility in the selection of agents for clinical trial. There was no thought at the time that these were necessarily the best or that they would be used indefinitely in the Center screen. Last summer the Center started a program in which about 60 different tumors were surveyed: human tumors, various tumors in Dr. Sugiura's spectrum, a group of about 20 hamster tumors in the hamster at Southern Research Institute, and others. It is hoped that other test objects with interesting, desirable characteristics will emerge.

Table 2.—Representative tumor spectrum data

	Inhibition of various tumors							
Compounds	1	2	3	4	5	6	7	8
A	−	++	−	−	−	−	−	−
B	−	+	−	++	++	−	−	−
C	±	−	−	+	−	+++	+++	+++
D	±	±	−	++	−	+++	+++	+++
E	++	+	±	−	++	+++	−	+++

As mentioned, Sarcoma 180 was selected because of certain characteristics; unfortunately we cannot rely on these characteristics remaining constant. Table 3 indicates, as shown by the lower figures in the SRI column compared with those in the SKI column for inhibition of growth by various compounds, that the SRI tumor, derived from the SKI line several years prior to these data, had retained a sensitivity, whereas the SKI line had become somewhat more resistant to the therapeutic agents (*8*).

Table 3.—Comparison of tumor retardations in two lines of Sarcoma 180

Compound	Relative tumor diameters	
	SKI	SRI
Diaminobiuret	0. 79	0. 63
Sulfomerthiolate	0. 78	0. 48
6-Mercaptopurine	0. 81	0. 55
Netropsin	0. 81	0. 64
TEM 0.75 mg.	0. 48	0. 32
TEM 0.50 mg.	0. 66	0. 42
9,10-Dimethyl-1,2-benzanthracene	0. 80	0. 58

Table 4 shows an interesting observation of Dr. Biesele and Dr. Hutchison with some of the resistant leukemia lines of L1210, including the original line, the lines resistant to amethopterin, mercaptopurine, fluorouracil, and so on. A number of resistant leukemias, besides those that were isolated primarily with resistance to amethopterin, also showed resistance to amethopterin. An interesting correlation was observed: Those lines resistant to amethopterin had lost the submetacentric chromosome *(9)*.

Table 4.—Resistant lines of L1210 leukemia

Leukemia line	Chromosome number of modal cells			Resistance to amethopterin
	Telocentric	Submetacentric	Minute	
L1210	38–40	1	1	—
L1210/A	41		1	+
L1210/A	40			+
L1210/MP/A	40			+
L1210/MP	40			+
L1210/AZS	40		1	+
L1210/FU	40	1		—
L1210/FU	39–40	1	1	—
L1210/MP	38	1	1	—
L1210/MP	38	1	1	—

The Center recognized early in the program that there might be genetic changes and variations in the test objects and, as a result of this need for controlling or at least monitoring the biological test material, Dr. Amos, of The Roswell Park Memorial Institute, was asked to investigate the aspects of genetic variability in the tumor-host relationships of the L1210 and Adenocarcinoma 755 systems. With his permission *(10)*, I can state that there have been gross breaches in the strain specificity of some of the samples; of about 70 that were sampled, 3 of L1210 and 1 or 2 of Adenocarcinoma 755 did show a marked loss in the host-specificity requirement. Several of those samples of L1210 also showed a marked deviation from the characteristic cytologic pattern. Of course, these were discarded from use in the national program.

Dr. Amos also found that there were deviations from the genetic background of some of the host animals, for some of them failed to take tumors which have this host-specificity requirement. Again, this was corrected. Also, a more definite examination of the antigenic characteristics of the tumors was made and again deviations appeared in the tumors that were being used in the program. This suggests the need for stricter control of the test material.

We are frequently faced with a worn-out question: Are we trying to cure cancer in mice or men? Actually it was with this difference in mind that Sir Charles Dodds, at the Symposium held at Memorial Center (*11*), suggested that materials be tried directly in man and that intensive studies be carried out to provide material and information for leads for further developments. This suggestion was made, of course, with the stipulation that the welfare of the patient be kept foremost in mind in all these studies. Useful as this type of study might be, it does not eliminate the need for supplemental information from screening programs. We simply do not have enough potential in the direct human study to cover the things that need to be examined.

Dr. Handler (*12*), Dr. Woolley *et al.* (*13–16*), and the Pfizer group, as part of the national program, have been examining the human tumor in various hosts. Figure 1 shows the human cancer in the cheek pouch of the hamster. I have reservations on the utility of a human tumor grown in a heterologous host, but I think we should "be from Missouri" and be shown. If these test systems bring forth even one good material not developed by other test systems, they would be worth the cost and effort.

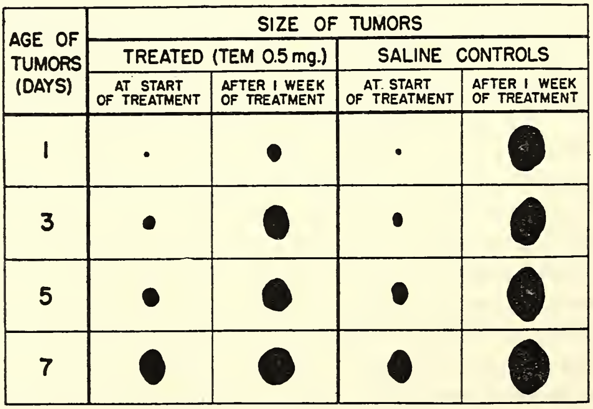
With the increasing interest in viruses that has been developing over the last few years, we have introduced into Dr. Sugiura's spectrum the Friend virus leukemia (*17*). In figure 2 the appearance of a mouse with leukemia is compared with a normal mouse. The difference in the size of the spleen is to be noted. This is usual for a 3-week observation. We have tentatively adopted a screening program by evaluating the spleen weight at the end of 3 weeks. Figure 3 shows spleens from infected mice treated with an effective agent and spleens from untreated, infected mice. The spleens in the latter are about tenfold the weight of the normal spleen. These treated spleens have been prevented from enlargement by administration of various of the alkylating agents. The alkylating agents have been the most effective in this system; however, the better purine antagonists have been nearly as effective (*18*). Of course, when the effects are good it is necessary to follow them with titrations of the spleen for virus and for observations on survival time. We have not used survival time routinely in the screening because it is not a very constant characteristic.

Questions also have been repeatedly raised about the use of the spontaneous tumor, because that is the type of disease in man. In the use of transplanted tumors there are possibilities for additive effects from immune mechanisms. We have evidence from our experimental studies that immune mechanisms play a role in some of the effects that we see (*19*). Table 5 contains data from studies with the RC adenocarcinoma in the mouse. Of 110 animals in the control group, only one regression occurred. With the particular dose schedule of mercaptopurine, there were 53 regressions out of 110 animals. We tried to demonstrate that immune mechanisms were aiding in the development of the damage done by the chemotherapeutic agent. Cortisone was given to animals treated in the same way with 6-mercaptopurine and we did show a drop in the number of regressions from 53 to 14 out of 110. I do not think the added effects due to immune mechanisms should be a matter of concern. I think it actually can help us identify some of the smaller degrees of chemical damage.

Table 5.—Effect of cortisone on 6-mercaptopurine-induced regressions of the RC adenocarcinoma

			Result at 2 weeks		Result at 8 weeks	
Treatment	Dose (*mg./kg./day*)	Tumor age	Deaths	Average tumor diameter (*mm.*)	Deaths	Regressions
Control			17/110	18. 5	109/110	1/110
6-Mercaptopurine	15	1	2/110	6. 5	55/110	53/110
6-Mercaptopurine + Cortisone	15 50; 25	1 12; 16	1/110	6. 9	91/110	14/110

Some of those who have been interested in the spontaneous tumors have cited the fact that one cannot hit the spontaneous tumors as readily as the transplanted tumors. I think some of these statements are based in part, however, on comparisons with what can be done against the early transplanted tumor, such as that shown in text-figure 2, compared with the control. If we make the comparisons with the tumors in which there has been delayed treatment, *e.g.*, for 7 days, then we find it more difficult



Text-figure 2.—Variation in Sarcoma 180 inhibition with delay in treatment.

to hit the well-established transplanted tumor. This is sometimes overlooked in that some fail to recall that the spontaneous tumor also is a well-established tumor and the comparison should be made with what can be done with a well-established transplanted tumor.

As was indicated, the three-tumor screen was adopted in the national program because of differences among tumors in their responses to agents. The use of a tumor spectrum has been considered for a number of other reasons:

1. Detection of screening misses.—A good example of this, though it did not prove to be a useful agent for treatment, but nevertheless illustrates a point, was that 8-azaguanine would have been missed by many of the experimental tumors (*20*). It was picked up by certain mammary tumors. This is a theoretically interesting compound. Therefore we believe that various compounds that have theoretical interest should be examined in a tumor spectrum in order to detect possible activities that might be missed in the tumor screen.

2. A comparison of related activities.—This consideration will be referred to later.

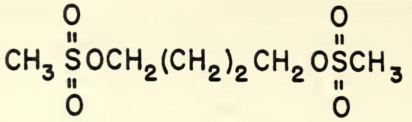
3. A more complete description of the actives.—Some clinicians wish to know how broadly active against a spectrum of experimental animal tumors a substance may be. Others, on the contrary, may be interested in a high degree of specificity of action, something that hits only one or two out of a spectrum. Nevertheless, many clinicians desire the background information on how broadly active a compound may be.

4. Specialized assay tools.—When cortisone first became available, we tested it in the tumor spectrum and found that, as had been demonstrated by Heilman and Kendall (*21*), the lymphosarcomas in the mouse are unusually sensitive, as also are several of the so-called osteogenic sarcomas. Accordingly, for the corticosteroids we used osteogenic sarcomas or lymphosarcomas in the spectrum (*22*).

The actinomycins were not very effective in the standard screening test against Sarcoma 180, the tumor that we were using as our main screening tumor (*23*). [*See* activity against Sarcoma 180 under certain test conditions (*24*).] However, they produced desirable effects against the Ridgway osteogenic sarcoma and against certain mouse leukemias that Dr. Burchenal has used. In general, for routine screening, we have used the Ridgway osteogenic sarcoma because it not only demonstrates the activity of the actinomycins but enables us to fashion a certain degree of therapeutic index for various actinomycins (*25*). It is also possible that a more sensitive tumor could be found in a spectrum as an assay tool for a weak culture-filtrate activity detected by a screening tumor.

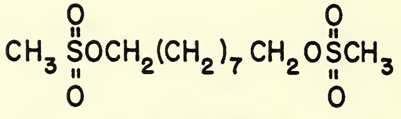
5. Development of new screens.—We have considered this to some extent previously, and it may be that through compiling enough information of the tumor-spectrum results a useful new object for screening purposes may be found.

The formulas of methanesulfonic acid, tetramethylene ester (Myleran) and of the nonane analogue are shown.



$$CH_3\overset{O}{\underset{O}{S}}OCH_2(CH_2)_2CH_2O\overset{O}{\underset{O}{S}}CH_3$$

1,4-di (methane sulfonoxy) butane



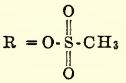
$$CH_3\overset{O}{\underset{O}{S}}OCH_2(CH_2)_7CH_2O\overset{O}{\underset{O}{S}}CH_3$$

1,9-di(methane sulfonoxy) nonane

Table 6 demonstrates, in a limited spectrum, that if we had tested against certain of these tumors there would have been no difference between the two compounds, tested at maximum tolerated doses. With several other tumors, however, there is an indication of a possible superiority of the nonane analogue over Myleran.

Table 6.—Comparison of dimesyl alkanes against mouse tumors

Compounds	Ehr.	C1025	GE	WOS	GLS	HP
R-(butane)-R	±	+ to ++	—	—	—	±
R-(nonane)-R	±	++	++	++	± to +	±



$$R = O\text{-}\overset{O}{\underset{O}{S}}\text{-}CH_3$$

In the program of the Center, tissue culture has a definite role as a possible screening tool and has been used for primary screening. Eagle and Foley, in their original report (*26*), indicated that of the highly cytotoxic agents about 80 to 95 percent, dependent on the degree of cytotoxicity, were also antitumor agents as judged by the information in the literature. Dr. Schepartz, in the Center, surveyed the data obtained by the Center and compared the tissue-culture results with the three-tumor screening results. This comparison indicated that, in the animal tumor programs, if we were to screen only those materials which had a high degree of cytotoxicity there might be a fivefold to ninefold enrichment of the yield (*27*). In other words, if we took those materials which were cytotoxic at about 1 to 10 μg. per ml. in tissue culture, we would have to test only one fifth to one ninth as many materials in the animal tumor screen to obtain a given number of active materials, that is, materials proved active by the criteria of animal tumor screen but as yet untested in man. Dr. Skipper and Dr. Schabel, of the Southern Research Institute, independently came to similar conclusions based on their data (*28*).

Another tissue-culture assay, originally developed at Parke, Davis & Company (*29*), is being investigated in several aspects of the national program by the Pfizer group (*30*), the group at Southern Research, and others. It may be a simpler, more economical method that will give similar results.

Bacteria can be useful. Even before the surveys on the tissue-culture results, Dr. Zybalski, in a screening conference held by the Center over a year ago, showed that if the strongly mutagenic materials were examined (table 7) 22 of a total of 431 materials were strongly mutagenic and 369 were nonmutagenic; of the strongly mutagenic, 86 percent had antineoplastic activity, whereas only 7 percent of the nonmutagens had antineoplastic activity (*31*). This was based on the animal tumor screening results in the literature. This suggests a possible prescreening device by which we could enrich the ore of the materials that we look at in the animal tumor screen.

TABLE 7.—Antineoplastic activity of mutagenic and nonmutagenic substances

	Mutagens		Non-mutagens	Total
	Strong	Weak		
Number of substances	22	40	369	431
Percent of total	5%	9%	86%	100%
Number with antineoplastic activity	19	18	25	62
Percent in category	86%	45%	7%	14%

Dr. Hutchison, in studies with *Streptococcus faecalis*, saw the development of a collateral sensitivity [table 8, (*32*)]. In the wild type with a sensitivity to amethopterin, when the amethopterin resistance is developed, a much smaller amount (1/10 as much) of the 6-MP is required. This certainly suggests extension of the use of this rather special screen to try to find materials that might be more useful in patients who are resistant to a standard agent.

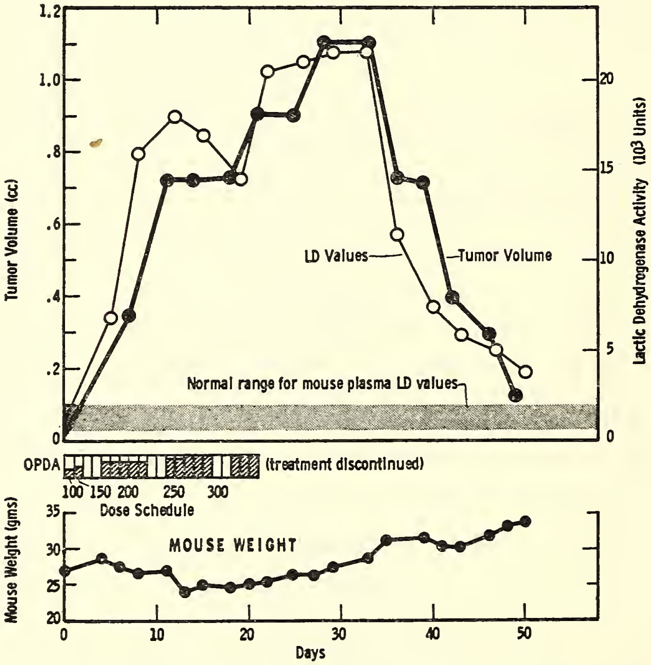
TABLE 8.—Sensitivity of *Streptococcus faecalis* to amethopterin and 6-mercaptopurine

Culture	μ per ml. for 50 percent inhibition	
	Amethopterin	6-MP
SF/0*	0. 0005	10
SF/A†	60	1
SF/MP‡	0. 003	>500

*Wild strain *Streptococcus faecalis.*
†Amethopterin-resistant *Streptococcus faecalis.*
‡6-MP-resistant *Streptococcus faecalis.*

In the national program there are a number of active materials appearing from culture filtrates. In addition to the microbial assays and the physical measurements, we need other techniques to help identify possible duplicates. For example, in the embryonated egg the growth of the embryo is affected by azaserine in a specific way. Dr. Karnofsky has described the more detailed aspects of the abnormalities (*33*). It is sufficiently characteristic that it has been helpful several times as an aid in identifying as an azaserine- or a DON-related material some of the culture-filtrate actives.

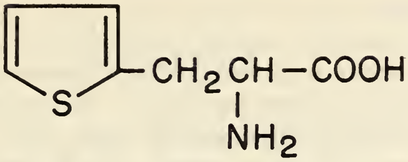
It will be useful to have any techniques that will reveal as quickly as possible what is happening in our chemotherapy treatment. In text-figure 3 is indicated the tumor volume by the solid lines; the tumor is regressing as a result of treatment with *o*-phenylenediamine (*34*). This shows perhaps too good a parallel with the results of the tumor growth, but it indicates the possibilities, perhaps both in animals and in man, of an early detection procedure based on a blood sample as to what is happening to the tumor under treatment.



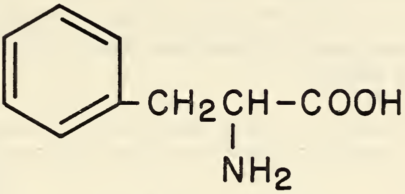
TEXT-FIGURE 3.—Correlation of tumor growth and regression with lactic dehydrogenase activity in blood plasma. The *light lines* and *open circles* indicate lactic acid dehydrogenase values from the blood.

Figure 4 shows how we can obtain these frequent blood samples by the use of a capillary tube into the corner of the eye of the mouse (*35*).

Dr. Baker mentioned the transaminases. The formulas of phenylalanine and one of its antimetabolites, β2-thienylalanine, are illustrated.



β-2-Thienyl alanine

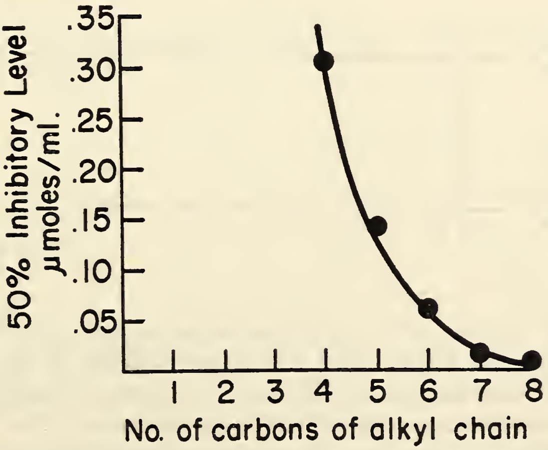


Phenyl alanine

Figure 5 shows a tissue-culture preparation in which the antimetabolite was present. In the tube at the right can be seen the pigment that has developed. This tube contained normal mouse tissue implants. The color developed as a result of the ability of that tissue to use β2-thienylalanine in transamination.

The interesting thing to us is shown in figure 6; the tumor tissue has lacked this particular enzyme, this transaminase (*36*). We are still trying to make this a useful material, possibly by building it into polypeptides.

Our interest in trying to interfere with protein metabolism has been additionally stimulated by some results that we saw with some of the esters of L-tryptophan (*37*). Text-figure 4 shows the increasing effectiveness of the esters of tryptophan with the length of the carbon chain



Text-figure 4.—Effect of the alkyl chain length of esters of L-tryptophan on the growth of *E. coli*.

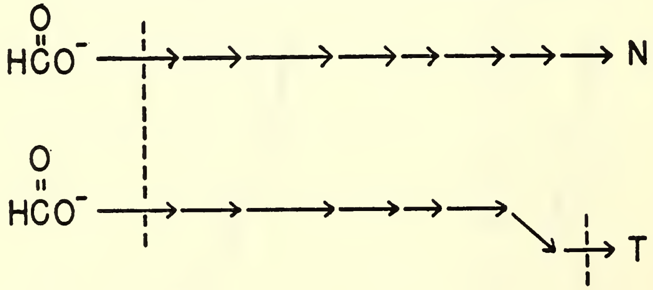
against *Escherichia coli*. We hope that this is interference with protein metabolism and that this may be a lead which we can follow.

Though my report may not be very encouraging, several clinically useful compounds are shown in table 9. We do know that up to several years ago practically all the materials which were useful in clinical trials were first detected by experimental animal studies (*38*). (Exceptions are potassium arsenite and the androgens and estrogens.) However, we cannot be complacent about that. We have to keep examining our test systems because, as you know, none of these compounds are really curative. It may be that our test systems are not at fault. We may be simply testing the wrong things.

TABLE 9.—Compounds used for treatment

Clinically useful compounds	Date of experimental report
Methyl-bis(β-chloroethyl)amine	1942
Triphenylethylenes	1942
Cortisone	1944
Urethan	1946
Aminopterin	1948
Amethopterin	1948–9
Hydrocortisone	1950
TEM	1950
Myleran	1950
TEPA and STEPA	1952
CB1348	1952
6-Mercaptopurine	1953
6-Chloropurine	1954
Thioguanine	1954

The hypothetical pathways from formate to normal and cancer tissue nucleic acids or nucleoproteins, as a simplified series of parallel steps until very near the final steps, are shown. It is suggested that what we



are doing with agents, such as 6-mercaptopurine and amethopterin, is to cut across both the normal and tumor metabolism in the early stages without much discrimination. Perhaps we have to consider a macromolecular chemotherapy and thereby try to attack the late steps in metabolism where we can have an increased opportunity for a selective action by possibly a modified nucleic acid or nucleoprotein.

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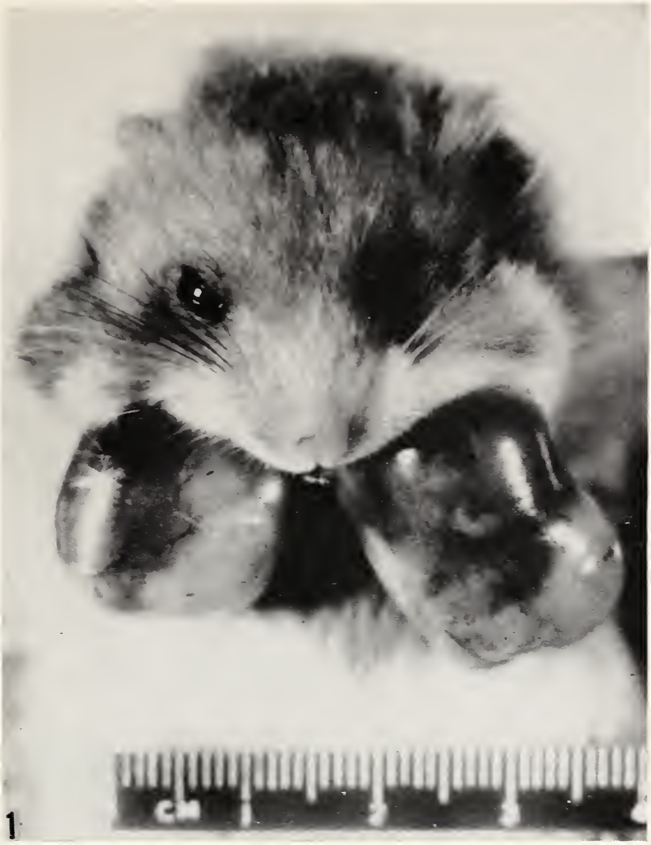
Plate 1

Figure 1.—Human cancer in cheek pouch of hamster.

Figure 2.—Mouse with leukemia compared with normal mouse.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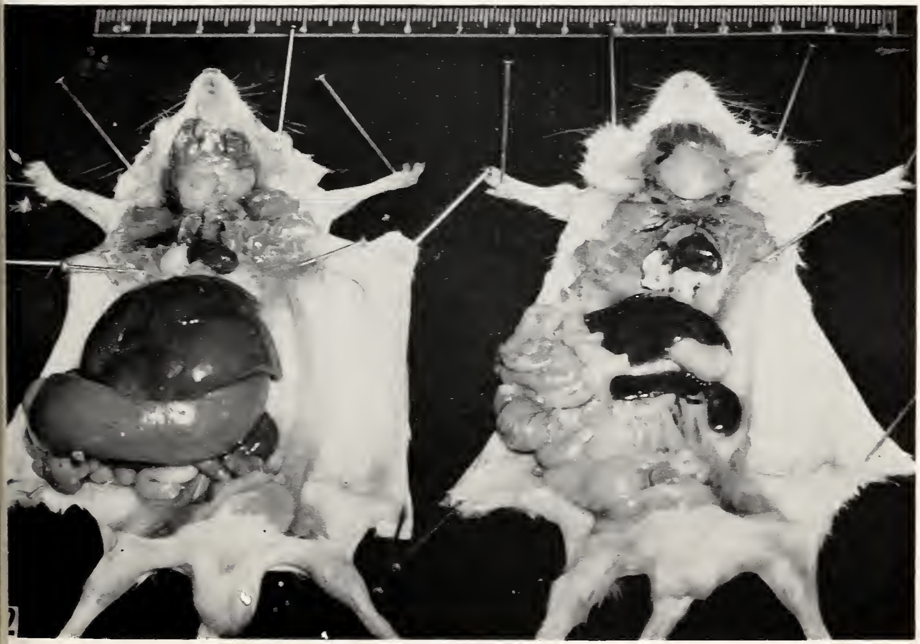
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PLATE 2

FIGURE 3.—Spleens from infected mice treated with effective agent compared with spleens from untreated, infected mice.

FIGURE 4.—Method used to obtain blood sample from eye of mouse.

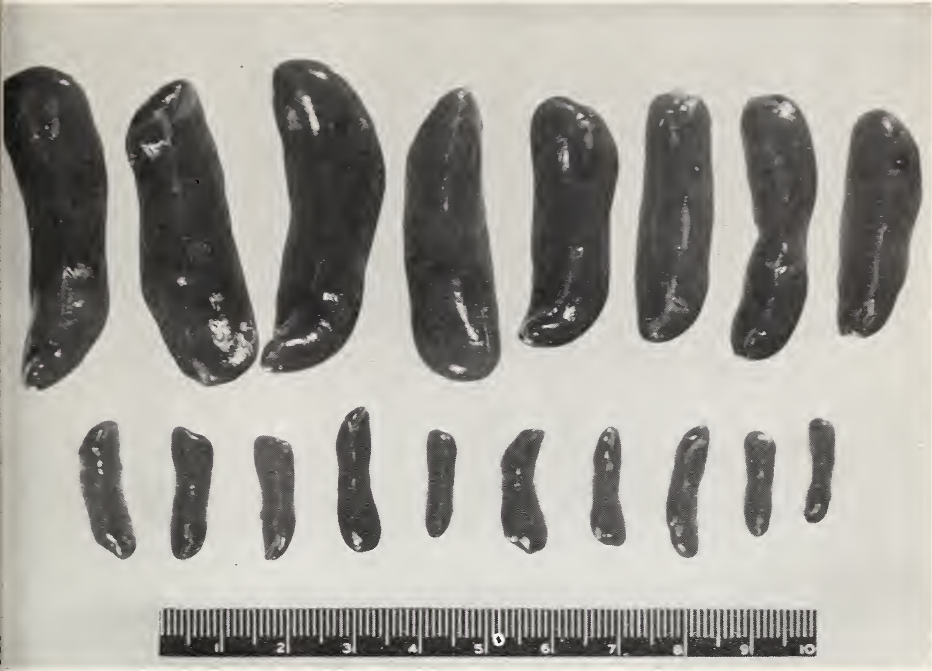
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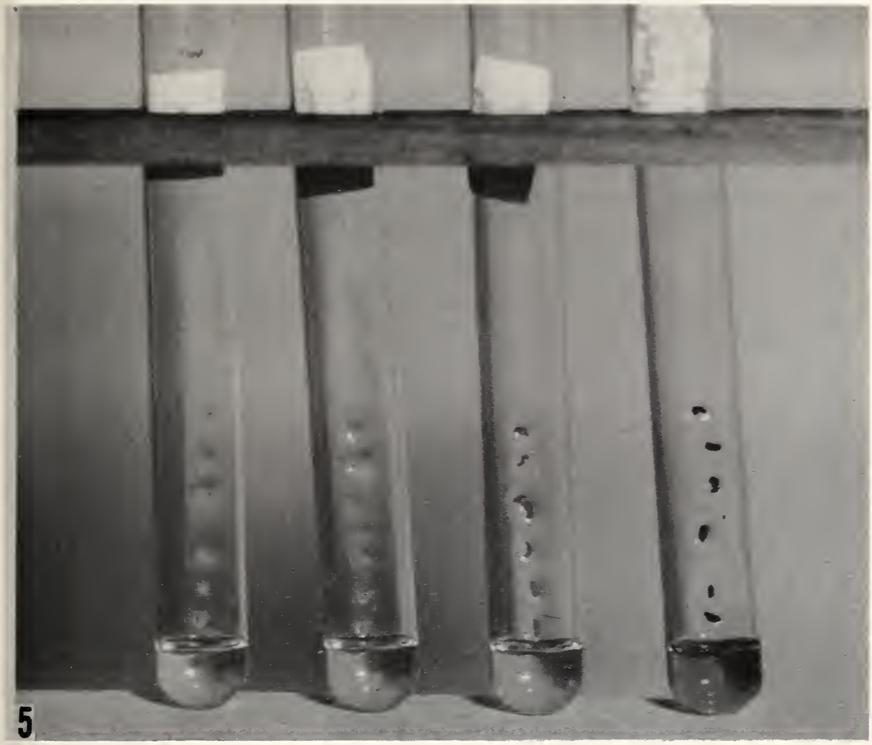
PLATE 3

FIGURE 5.—Tissue-culture preparation with β2-thienylalanine present.

FIGURE 6.—Tumor tissue lacking enzyme for transamination.



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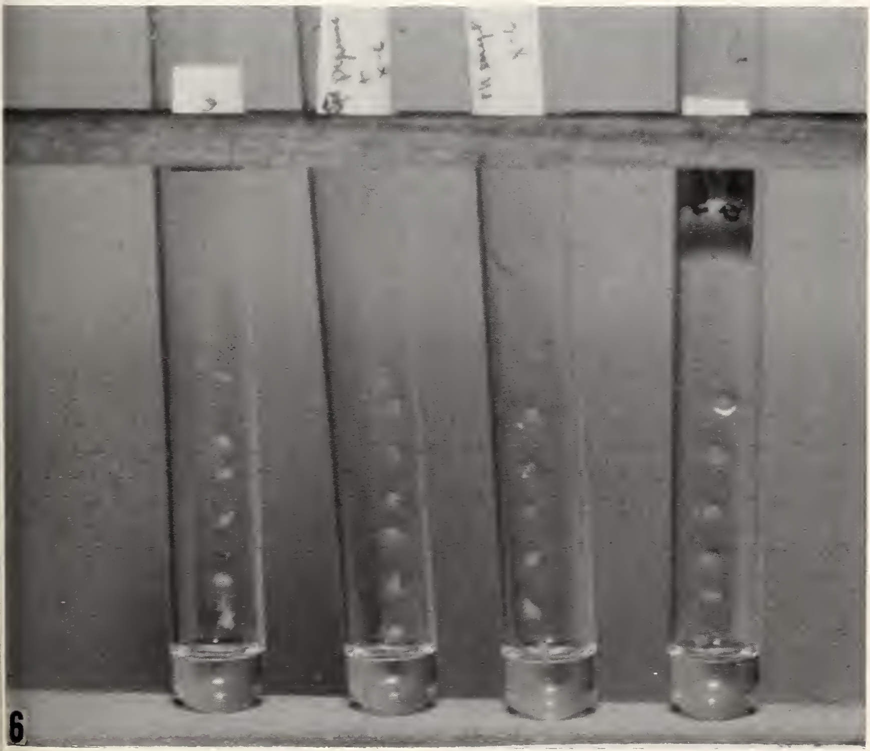

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DISCUSSION

Dr. Zeisler: I wish to compliment Dr. Stock and the Sloan-Kettering Institute on the extremely careful methods they have used to screen new compounds in chemotherapeutic agents for cancer. Unfortunately, up to the present time nothing has been discovered by the synthetic chemistry which offers any hope to the patient with Hodgkin's disease or leukemia. These are still classified as incurable diseases.

About a year ago, one of the large pharmaceutical firms obtained a chemical agent from plant leaves, not from the soil. The results of the tissue-culture tests and on three types of solid tumors in the mouse, including one type of leukemia, were reported to me as negative. I presume I should have been discouraged by this report. On the contrary, I was encouraged. I fully agree with Dr. Charles Huggins, who looked upon animal cancer tests as a necessary evil. He does them just to satisfy the medical profession, not to satisfy himself. What I am working with is a new type of a selective plant hormone, which I have called lyalphaline because it is obtained by the lyophilization of grass exudations of a fluid of different species of grasses that I can obtain in my own back yard and in which I have studied the organic constituents at great length and at great expense.

I am not sure at all that I have a solution for any of the present problems in the field of the lymphomas, but it is quite possible I may have. I am hopeful, however, that in the near future at some of our large institutions, such as the Massachusetts General Hospital or at the Memorial Center for Cancer, where they have a great deal of this material and are simply waiting for a patient to enter in the terminal phase of relapse, that some consideration will be given to a new method of approach for the treatment of diseases that are completely and totally incurable today.

Dr. Stock: I am quite prepared to find something that is not active in experimental animal tumors that may be active in man.

Question: I noticed from Dr. Stock's diagram that the last useful drug for therapy in man was developed several years ago. Could you tell us how many drugs, in the interim period, have shown in animal screening that they might be potentially useful?

Dr. Stock: This is difficult to answer. Would you consider 6-mercaptopurine or actinomycin as the last useful drug? In the national program we are trying to refine the list of compounds that have shown activity. In our own laboratory, we have, if I may start with the culture filtrates first, about 150. We do not know how many of those are duplicates because they have not progressed to the point that we know what they are, but we have 150 cultures which are in the various pharmaceutical companies' laboratories. They have been sent to us to test. But there are 150, minus whatever number of duplicates there may be, that have shown a definite reproducible activity; some of these are weak. Perhaps we will be lucky if 10 or 15 of that group can be useful for treatment in man. I have not seen the data of the national program on the number of active culture filtrates. Perhaps someone from the Center can indicate that.

Reply: We have about 20 or 30 compounds in our own laboratory which have shown a weak but reproducible activity. Some of these we are examining in other tumors to find out whether they show enough additional activity for clinical use. In the national program, there will probably be about 100 compounds when we have defined the criteria to indicate that these are active. A number of these will be closely related to other compounds; some are close relatives of 6-MP; some are related to the alkylating agents.

Dr. Handler: Dr. Stock, I understood you to say that you had some reservations about the use of human tumor in animal transplantation. I wonder if you would care to mention these at this time.

Dr. Stock: I would have no reservations if the human tumor in the heterologous host would demonstrate a utility that is unmatched—in other words, a detection of a material that would be useful in man which would not be identified by other methods. Those of you who use the system, and Dr. Handler himself would be one of those who would be best able to say, know that the human tumor in the heterologous host is a more expensive and tedious test object than that of the experimental animal tumor. I do not know what the percentages are now, but in the early stages it was only a small percentage of tumors that were taken from man that would grow in the heterologous host adequately for chemotherapy screening purposes. The question arises whether those that grow quite satisfactorily are truly representative of the human tumors. In addition, the conditioning required, usually with radiation and cortisone, makes a special host necessary and, in some instances, seriously reduces the amount of drug that the host will tolerate, again jeopardizing the detection of activity. One difficulty that has been somewhat overcome with a number of the human tumor lines, both by the work that Dr. Woolley's group has done and by groups in Boston, has been the rather unsatisfactory uniformity in percentage of growth and percentage of takes in the human tumor lines. There are now several which do quite well. These are some of the objections I have, but these will be wiped out if the human tumor in the heterologous host will demonstrate that it can clinically absorb useful compounds.

Planning Clinical Chemotherapy Studies[1]

LOUIS C. LASAGNA, *Division of Clinical Pharmacology, Johns Hopkins Hospital, Baltimore, Maryland*

There are several phases in clinical chemotherapy studies that deserve emphasis: (1) the choice of drugs for clinical trials. Despite the large number of patients with cancer in this country, clinical material is too precious to be wasted in the pursuit of false leads. Therefore, it is incumbent upon investigators to select compounds on which there are reliable and promising data in animal tumor screens and on which there are reliable data on animal pharmacology and animal toxicity. Investigators in cooperative groups should avail themselves of the expert advice available at the Cancer Chemotherapy National Service Center in checking upon efficacy and toxicity data at the preclinical level.

(2) It behooves us, whenever possible, to study drugs that will provide gains in two respects: data in regard to the efficacy of the drug *qua* drug and data bearing on the utility of the theoretical concept or tumor screen responsible for suggesting the drug. It would be most helpful to clinical groups if chemists and people concerned with preclinical screening, instead of continuously mumbling in their beards about the lack of clinical information to correlate with their data, would describe specifically what they would like as feedback from the clinical program, so that we may correlate best the data from preclinical screens and the information from patients.

(3) There is an unfortunate tendency, natural but nevertheless unfortunate, to rely excessively, in studies of preliminary toxicity or of efficacy, on "last-ditch" patients, so critically ill and so close to death that they provide little in the way of reliable information regarding toxicity or clinical efficacy. Such patients may be unresponsive to drugs or responsive in a peculiar way, which is misleading when such response is used to generalize other types of patients.

All of us know that cancer is probably not one disease. We are also aware that there is a spectrum of disease ranging from tumors more or less responsive to agents that we now have to tumors unresponsive to most of the drugs we have. It is tempting to study patients with disease entities that may be expected to show response and yet it is dangerous to concentrate on patients of this type for two reasons: (*a*) Most of the clinical material available to us for studying new agents is not in this category. (*b*) The great therapeutic challenge is not in the area of responsive tumors, such as lymphomas, but in the area of less responsive "solid tumors," such as cancer of the lung.

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[1] Presented at Conference on Experimental Clinical Cancer Chemotherapy, Washington, D.C., November 11, 1959.

(4) In details of drug administration, let us first consider dosage regimen. We know that one can observe differences in the toxicity and efficacy of anticancer drugs by alteration of the spacing of doses. This is true in animals and, at least for toxicity, in human beings. In planning drug studies, investigators ought to be aware of this and when possible include both "intermittent" dosage and "continuous" dosage schedules on the chance that an agent may be more effective one way than the other. This poses problems in regard to the amount of clinical material available and the amount of time that is to be spent on a study, but the principle ought to be kept in mind.

One might make the point that a remarkably efficacious agent would probably be picked up no matter how one gave it, but I am enough of a pessimist about our finding such an agent in the near future to believe that we will need to squeeze as much information as possible out of our studies, by utilization of different dosage regimens, for some time to come. If a group is to use only one dosage regimen, one would hope that the choice would be based on information from animal data, suggesting that that type was most effective. It is not always possible to transfer conclusions from animal to man, yet one would hope that there would be at least a partial correlation between animal and human data.

(5) In cancer chemotherapy, one of the most pernicious concepts is that of "maximally tolerated dose." This concept has come into being because of our frustrating situation in regard to the lack of good therapeutic agents. Faced with a drug that has little or no therapeutic activity, investigators are naturally reluctant to discard the agent without having pushed the drug hard enough to avoid the accusation that they merely did not give enough of the agent to see an effect. Yet the concept of maximally tolerated dose and the details of its definition vary considerably from investigator to investigator. For example, some investigators are made unhappy by significant marrow depression, whereas other investigators feel that serious marrow depression is worth risking if it gives significant antitumor effect. All of us want "optimal dose" rather than "maximally tolerated dose," *i.e.*, we want the dose that gives maximal efficiency at the lowest price possible.

In getting ready to study a drug, even after preliminary observations with the agent, we do not *know* the optimal dose because otherwise we would not have to perform the study. What we have, instead, is a dose that is a guess, based on early toxicity data. There are several possible outcomes after such a conjecture is made. One is that we may have guessed correctly in regard to the maximal tolerated dose. This is fine *if* the maximally *tolerated* dose is the maximally *efficient* dose, but it is not necessarily true in animal tumor experiments, and if you think of pharmacology in general outside of cancer chemotherapy, you will quickly realize that the maximally tolerated dose is not the optimal dose in many other areas of pharmacotherapy.

Having made an error in that the maximally tolerated dose is not the maximally effective dose, we may erroneously discard an agent. We may also guess too *high* at this "maximally tolerated dose," and for a variety of reasons: The criteria of a given investigator or group may be rather reckless, or the criteria may be respectable enough but difficulties may arise due to sampling problems, which may be serious in early experiments with drugs. The number of patients studied in such early probings is often fairly small, and such patients are often very sick people, so that one may make a bad guess merely because these patients are an inadequate sample of cancer patients in general. We may also guess too high for another reason: our inability to pick up the kind of toxicity that is not grossly obvious. It is easy enough to pick up hematopoietic depression, oral ulceration, or abdominal pain, but what about nonspecific "fading away" of patients, for example, in the Veterans Administration surgical adjuvant study, where it seemed that more patients died after alkylating agents in the immediate postoperative period, and yet an analysis of the types of deaths did little to explain the mortality. This phenomenon is reminiscent of the observation that in tumor-bearing animals treated with alkylating agents the animals may die at a rate more rapid than that of their control groups, without hematopoietic depression being present. This type of error may again make us guess too high at the "maximally tolerated dose." The result will be to discard a drug erroneously as ineffective. We may also guess too *low* at the "maximally tolerated dose," either because of poor sampling or because of ultraconservative criteria of tolerability. The result here also may be to discard a useful drug as ineffective.

A suggested solution is the classical pharmacological concept of using several doses in a study. The use of several doses will allow (at least more often than the use of a single dose) for the visualization of trends in regard to toxicity and efficacy and for the extrapolation of dose-response curves beyond the particular points chosen for study so that some guesses can be made about what would happen if we used higher or lower doses than the ones selected.

(6) Only a fool or a fanatic would insist that controlled trials, as they are usually defined, are necessary under all circumstances in this, or indeed any other, area of therapy. The more predictable the disease, the less susceptible to bias the criteria of evaluation, the less one needs controls. For example, a neoplasm with essentially no spontaneous improvement and with death as the endpoint needs little in the way of control groups. The same may be true of a disease with a considerable degree of responsiveness to drugs but with little variation in response. We do, of course, need controls when bias is involved in evaluation of response, when there is great variability and unpredictability of response or of spontaneous change, or when we know little about the rate of spon-

taneous change or the rate of response to standard drugs—and this may be more often true than we think at the moment.

The point I am trying to make is that a doctrinaire approach to the use of controls in all situations or the absence of controls in all situations is unwise. I would like to suggest a type of protocol for evaluating new agents that I think is an improvement over the approach currently used. This might be called a combination of early clinical pharmacology and a therapeutic trial.

Investigators will always have to indulge in preliminary observations with new agents to get a "feel" for the compound. At the same time, human nature is such that no investigator, administering a new drug to a patient with a tumor that can be followed in some way or other, is going to shut his eyes as to whether therapeutic response has or has not been seen. It is unrealistic to demand this. There is also no reason why an investigator should not get the most information possible from a study of different dose levels, including the lower ones that he will usually be starting out with, because these lower dose levels may be just as effective or perhaps more effective than higher, more toxic dose levels.

What I am suggesting is a type of sequential design, in the sense that the emphasis on dosage is shifted as information comes in. The drug will of necessity be given in small doses to begin with and gradually raised until one gets some feeling for dose levels that are probably too high and those that are probably ridiculously small. In between these limits I suggest the investigator focus on two or three dose levels and accumulate enough cases at each dose level and for each tumor category so that he can draw conclusions with some confidence.

We will have to study adequate numbers of patients because the risks of drawing unsound conclusions from extremely small samples are too great. On the other hand, we will have to set arbitrary limits on the number of cases studied per tumor category and per dose level because no group will be able or willing to study enough patients to eliminate with a high degree of certainty the possibility that the agent is really a good one. In other words, we may have to settle for an "80 percent security" in our feeling that the drug is no good rather than a "95 or 99 percent confidence."

We will, in studies of this type, set arbitrary limits of performance that will have to be met before a drug is considered exciting enough to pursue further. For example, we may insist that a drug cause a 20 percent remission rate for a given neoplasm in a given number of patients. This approach should work well if we are faced with a large number of agents that are essentially inactive. The discarding of these compounds at an early stage should be reasonably effective by the approach described.

With compounds that look promising in such experiments, in most instances we will follow up leads with larger scale, controlled experiments in which the promising new agent is pitted against either optimal standard supportive therapy (including placebos) or the standard drug considered most useful in that particular situation. This may not be an optimal scheme but I think it is an improvement over some of the methods currently in vogue. The basic considerations I have discussed should be subjected to frequent reappraisal, lest we settle into a complacent state and assume that we are functioning optimally, whereas, in fact, we may be working at a rather inefficient level.

DISCUSSION

Dr. Olson (Albany): In the discussion of planning clinical studies there was no mention made of remission time or survival time. We are now reaching the point where we ought to evaluate new agents on whether they are improving the treatment of cancer and improving the survival time. Many of the reports note remission of tumors, and we have a number of methods of measuring remissions of certain solid tumors. We do not know really whether we are prolonging life. The studies at our institution and Delafield Hospital indicate that nitrogen mustard does not prolong life. We do not have any good figures, for example, on the remission rate of nitrogen mustard and the duration of the remissions. Using nitrogen mustard as a control, do we have adequate figures to use it as a historical control to state that one alkylating agent is better than the other; is this a dose difference or a real difference; is it a better agent or a less toxic agent? We are at the point now where we ought to say, "Are we treating cancer patients any more successfully now than we were 15 years ago?" I feel from our own figures that we are doing perhaps more harm to certain patients with lymphoma than we did; I do not think they live as long as they did before, though they may be living with a little less pain. I would like to ask you for some help on the natural history of the disease and the remission rate of some of the older compounds that have been used a long time.

Dr. Lasagna: I agree that survival time is much more important than a transient unimpressive regression in tumor size and that a change in tumor size and prolongation of life are not always tied together. The problem of measuring survival time, however, is a serious one because so many of the neoplasms we are studying have significant variation in survival time, or survival time is long enough that a number of drugs are applied to the patient before death ensues. This makes it difficult to evaluate the effect of a single drug on the natural history of that particular patient. I think in certain situations, such as cancer of the lung—at least the cases of cancer of the lung that are available for cancer chemotherapy—the survival time is short enough that it can and should be used as a parameter of success.

Question: This is not really a question but simply support of Dr. Olson. With the vast amount of time, effort, and money being invested in this effort, it seems to me that we are not getting the most profit out of it by being satisfied with a single parameter of survival time. For example, in the area of lung carcinoma we are losing valuable clinical information by simply not recording it if only as a subprotocol—if you wish, call it unofficial—in this program; a vast amount of material is wasted because our single parameter is survival time.

Use of Medical Measurements to Predict the Course of Disease [1]

EDMUND A. GEHAN,[2] *Ph.D., Therapeutic Trial Section, Cancer Chemotherapy National Service Center, Silver Spring, Maryland*

THE stimulation for this paper was provided by the problem of estimating the prognosis for a particular diseased patient. Each time a patient sees a doctor, a number of medical measurements are made on this individual, such as determinations of age, sex, white blood count, and platelet count. The question arises: Can we use these medical measurements to predict the course of the patient's disease?

The physician, in making a prediction, would use his own experience with cases of a similar type and the experiences of others reported in the medical literature to come up with a subjective appraisal of the patient's chances. In this paper, I would like to present an objective method of doing the same thing. The data from a number of patients will provide a weight for each of the clinical factors of importance in estimating the prognosis of a particular patient.

To be specific, I would like to consider the use of 17 variables to predict the probability of a remission in a patient with acute leukemia. This problem was chosen because it can be clearly stated and because data were available from a trial conducted by the acute leukemia B cooperative group. The method used, regression analysis, is a well-known multivariate statistical technique and could be used on related problems.

The following 17 variables were used to predict the probability of a remission:

X_1 = sex: male = 0, female = 1
X_2 = age: child (less than 20) = 1, adult (20 or over) = 2
X_3 = type of acute leukemia: lymphocytic = 0, myelocytic = 1, unclassified = 2
X_4 = prior treatment: none = 0, steroids = 1, other = 2
X_5 = symptoms to diagnosis (weeks)
X_6 = diagnosis to treatment (weeks)
X_7 = treatment: methotrexate = 1, 6-MP = 2, combination = 3
X_8 = liver (cm.)
X_9 = spleen (cm.)
X_{10} = nodes: none = 0, less than 2 cm. = 1, 2 cm. or more = 2
X_{11} = log (white blood count)

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[1] Presented at Conference on Experimental Clinical Cancer Chemotherapy, Washington, D.C., November 11, 1959.

[2] The author wishes to thank Mr. Nathan Mantel for helpful discussions, Mr. Robert Brunelle for processing the data, and Mrs. Jackie Rosenthal and Miss Joyce Shorts for additional statistical computations.

X_{12} = polys (%)
X_{13} = blasts (%)
X_{14} = log (platelet count)
X_{15} = temperature: 0 to 6, low to high
X_{16} = hemorrhage: none = 0, mild = 1, moderate = 2, severe = 3
X_{17} = performance: 0 to 3, normal activity to 100 percent of time in bed.

Nine of the variables are qualitative, taking arbitrary values, and 8 are measured. The variables were chosen after consultation with investigators in the acute leukemia cooperative study group and were those that were considered to have a possible effect on whether or not a patient reached a complete or partial remission. Some mathematical assumptions underlying the use of regression analysis do not necessarily hold for this collection of variables. However, this can be considered a first approach where the regression equation is used as a descriptive tool for estimating the probability of remission for a patient with acute leukemia. There were case records available for 202 individuals in a comparative study of the effectiveness of 6-mercaptopurine, methotrexate, and a combination therapy of 6-MP plus methotrexate. The values of these variables were taken at the start of the study and on the nearest day to 2 weeks after the start of the study. The analysis of these data was then conducted in 2 parts:

(1) Using the values of the 17 variables at the start of the study for 202 patients. (This is called Study I or referred to as *start* study.)
(2) Using the values of $X_1, X_2, \ldots, X_8$ at the start of the study and using the change in the variables $X_9, X_{10}, \ldots, X_{17}$ over the first 2 weeks of the study, *i.e.*, the value for each of these variables was taken as (value at start) minus (value at 2 weeks). There were 168 patients available for this analysis. (This is called Study II or the *start—2 weeks'* study.)

The regression-analysis solution to the problem of finding the probability of a remission, with the information on the 17 variables given, consists in finding a set of 17 weights (called regression coefficients) and applying each weight to the appropriate variable to obtain a linear combination of the weights with the variables. The form of the solution is as follows:

$$\begin{pmatrix}\text{Probability}\\ \text{of a remission}\end{pmatrix} = (\text{constant}) + w_1\,(\text{sex}) + w_2\,(\text{age}) + \ldots + w_{17}\,(\text{performance}).$$

These weights are determined so that the deviation between remission status (designated *one* for remitters and *zero* for non-remitters) and predicted probability of a remission, when squared and summed over all individuals, will be as small as possible. This is a "least squares" solution and is a well-known statistical technique (*1*). Alternatively, a transformation of these data could have been made and a weighted regression analysis performed according to the variation in each of the variables. For a first approach, this more elegant solution was not done.

The predicting equation for estimating the probability of a remission for the study with 17 variables available at the *start* is as follows:

$$\begin{aligned}\left(\begin{array}{l}\text{Probability}\\ \text{of a remission}\end{array}\right) = {} & 0.82-0.28\ (\text{age})-0.09\ (\text{type})\\ & -0.0003\ (\text{blasts})-0.04\ (\log \text{WBC})+0.10\ (\text{sex})\\ & +0.04\ (\text{treatment})+0.02\ (\text{prior treatment})\\ & -0.001\ (\text{symptoms to diagnosis})-0.001\ (\text{diagnosis to treatment})\\ & +0.001\ (\text{liver})+0.005\ (\text{spleen})-0.06\ (\text{nodes})\\ & -0.001\ (\text{polys})+0.02\ (\log \text{platelets})-0.02\ (\text{temperature})\\ & -0.02\ (\text{hemorrhage})+0.002\ (\text{performance}).\end{aligned}$$

Thus, if a patient with acute leukemia were over 20 years of age, had lymphocytic leukemia, and asked an investigator, "What is my chance for a remission?" one could begin finding the probability of a remission as follows:

$$\left(\begin{array}{l}\text{Probability}\\ \text{of a remission}\end{array}\right) = 0.82-0.28\ (2)-0.09\ (0)+\ .\ .\ .\ .$$

The probabilities of remission were calculated for the 202 cases and the average probabilities were as follows:

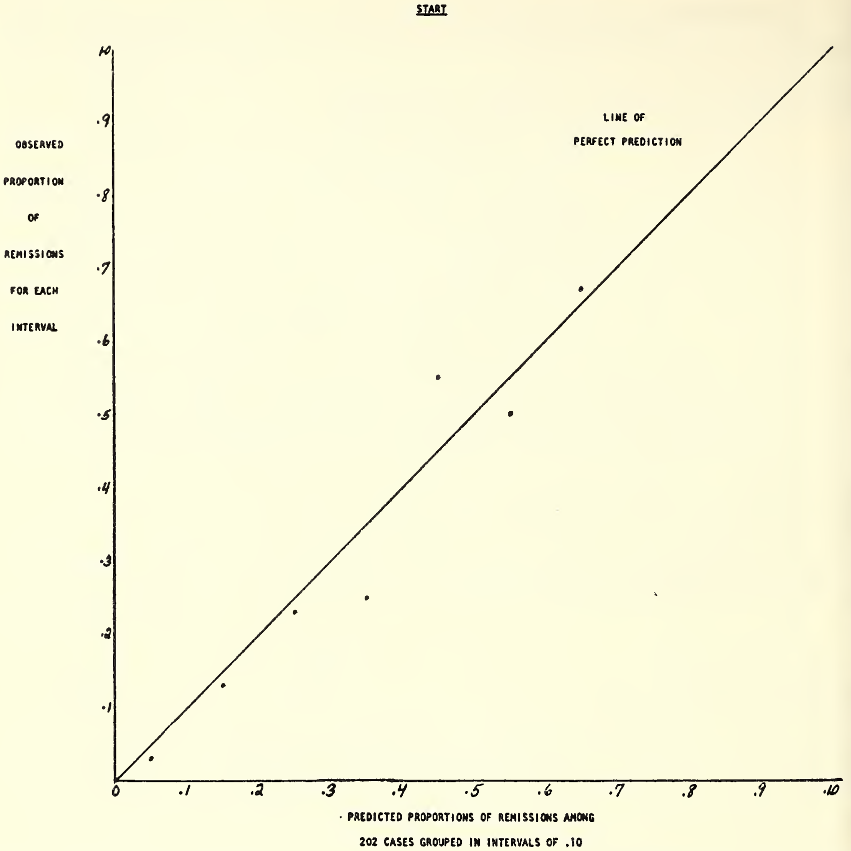
	Remitters (51)	Non-remitters (151)
Average probability	0. 41	0. 21
Standard deviation	0. 15	0. 20

Thus the probability of a remission is substantially higher in the remission group than in the nonremission group. The regression equation accounts for 20 percent of the total variation in remission status among the 202 patients, and the multiple correlation coefficient between remission status and predicted probability of a remission is 0.45. (Both these figures are statistically significant at the 0.1% level.)

The 202 predicted probabilities were grouped in intervals of 0.10, and the proportion of remissions actually observed was noted for each interval. These data are presented in table 1 and graphed in text-figure 1.

TABLE 1.—Frequency distribution of predicted proportion of remissions among 202 cases of acute leukemia and proportion of remissions observed for each interval

Start study				
Predicted proportion of remissions from regression equation	Cases	Remissions	Observed proportion	Expected proportion
Less than .10	52	1	. 02	Less than .10
.10–	38	5	. 13	. 15
.20–	22	5	. 23	. 25
.30–	28	7	. 25	. 35
.40–	38	21	. 55	. 45
.50–	16	8	. 50	. 55
.60–	6	4	. 67	. 65
.70 and over	2	0	0	Over .70
Total	202	51	. 25	



TEXT-FIGURE 1.—Proportion of remissions observed among 202 cases of acute leukemia grouped by probability of remission predicted from regression equation.

There is a close correspondence between the observed and the predicted proportion of remissions for each interval, except in the intervals where the predicted proportions were 0.30 to 0.40 and 0.40 to 0.50. In all except these 2 intervals, the observed proportion was within 0.05 of the predicted; in all intervals, the observed was 0.10 or less from the predicted proportion. There were no remissions in individuals with predicted probabilities over 0.70; however, this interval contained only 2 individuals. It should be emphasized that the regression equation was used on the same set of data from which it was derived; it should more legitimately be applied to some new cases of acute leukemia. The number of cases available did not permit this.

One might well ask the precision of the estimated probability of remission for an individual, *i.e.*, the standard deviation. The standard deviation for an individual depends upon his values of the 17 variables. The minimum standard deviation is 0.03, and the maximum standard deviation is about 0.12. Thus, at the outside, the estimated probability of a remission for an individual should be correct within 0.24 approximately 95 percent of the time.

The values of the individual weights in the regression equation can be ranked as to their importance in explaining variation in remission status. For Study I, the most important variables are ranked in table 2.

TABLE 2.—Variables from Study I (*Start*) ranked in order of importance

1. Age	Most important
2. Type of leukemia	
3. Percent of blast cells	Of lesser importance
4. White blood count	
5. Sex	
6. Treatment	

The regression coefficients of the other 11 variables did not explain additional variation in remission status, and these variables could have been dropped from the analysis. To a limited degree, the actual regression weight can be used to indicate the magnitude of the importance of each variable. For example, if the values of the other variables remain the same, a child has a 28 percent better chance of a remission than an adult; a female has a 10 percent better chance than a male; a lymphocytic leukemia patient has a 9 percent better chance than a myelocytic patient; an individual with a white blood count of 10,000 has a 4 percent better chance than an individual with a count of 100,000. The problem with this type of analysis is that it is difficult to conceive of changes in one variable where the values of the other variables do not change.

In Study II, in which the values of some variables were taken as (value at start) minus (value at 2 weeks), an attempt was made to use changes in the patients' status to provide an improved prediction of the probability of a remission. For example, perhaps the initial value of the white blood count is not too important for an individual: Maybe it is the change in count after the first 2 weeks of study. The period of 2 weeks was chosen arbitrarily as a period over which important changes in variables might be expected to take place.

The predicting equation for estimating the probability of a remission for Study II is as follows:

$$\begin{aligned}
\text{(Probability of a remission)} = {} & 0.62 - 0.27\ \text{(age)} - 0.08\ \text{(type of leukemia)} \\
& + 0.07\ \text{(performance)} + 0.06\ \text{(hemorrhage)} \\
& - 0.002\ \text{(symptoms to diagnosis)} + 0.05\ \text{(sex)} \\
& + 0.05\ \text{(treatment)} + 0.05\ \text{(prior treatment)} \\
& - 0.002\ \text{(diagnosis to treatment)} - 0.01\ \text{(liver)} \\
& + 0.01\ \text{(spleen)} + 0.02\ \text{(nodes)} - 0.005\ \text{(log WBC)} \\
& - 0.001\ \text{(polys)} + 0.001\ \text{(blasts)} + 0.001\ \text{(log platelets)} \\
& - 0.01\ \text{(temperature)}
\end{aligned}$$

The probabilities of remission were calculated for the 168 cases available for this analysis. The average probabilities were as follows:

	Remitters (51)	Non-remitters (117)
Average probability	0.46	0.24
Standard deviation	0.17	0.20

This regression equation accounts for 23 percent of the total variation in remission status, and the multiple correlation coefficient between remission status and predicted probability of a remission is 0.48. (Again, both figures are statistically significant at the 0.1% level.)

As before, the 168 predicted probabilities were grouped in intervals of 0.10 and the observed proportion of remissions was noted for each interval. These data are presented in table 3 and graphed in text-figure 2.

Table 3.—Frequency distribution of predicted proportion of remissions among 168 cases of acute leukemia and proportion of remissions observed for each interval

Predicted proportion of remissions from regression equation	*Start—2-week* study			
	Cases	Remissions	Observed proportion	Expected proportion
Less than 0.10	36	2	0.06	Less than 0.10
0.10–	28	3	0.11	0.15
0.20–	13	2	0.15	0.25
0.30–	22	5	0.23	0.35
0.40–	39	18	0.46	0.45
0.50–	15	11	0.73	0.55
0.60 and over	15	10	0.67	Over 0.60
Total	168	51	.30	

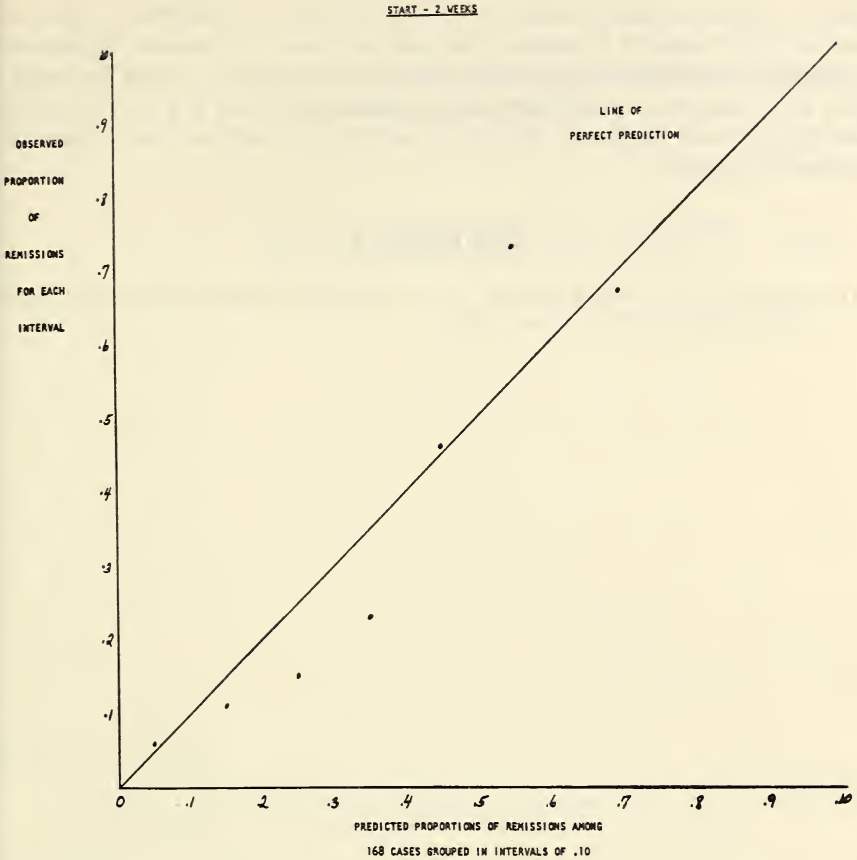
Again, there is a close correspondence between the observed and predicted proportion of remissions for each interval. The largest deviation from expected is 0.18 in the 0.50 to 0.60 interval. These predicted probabilities, while fairly good, are certainly no better than the predictions that could have been made at the start of the study.

The minimum standard deviation of the estimated probability of remission is 0.03 and the maximum standard deviation is about 0.12, the same as for Study I.

The ranking of the variables in order of the importance in explaining variation in remission status is given in table 4.

Table 4.—Variables from Study II (*Start—2 weeks*)

1. Age	Most important
2. Type of leukemia	
3. Performance status	
4. Hemorrhage	
5. Symptoms to diagnosis	Of lesser importance
6. Sex	
7. Treatment	



TEXT-FIGURE 2.—Proportion of remissions observed among 168 cases of acute leukemia grouped by probability of remission predicted from regression equation.

For this analysis, age and type of leukemia were again the most important determining factors in remission, a child having a 27 percent better chance for remission than an adult, and a lymphocytic patient an 8 percent better chance than a myelocytic patient. Change in performance status and hemorrhage were also important variables. The analysis reflects the obvious fact that the chances for a remission get better if a patient has improved his performance status and stopped hemorrhaging. The chance is 6 percent better for a patient who has stopped hemorrhaging and 7 percent better for a patient who improves his performance status by one category.

In summary, an analysis has been performed with the values of 17 variables to predict the probability of remission in a patient with acute leukemia. The predicted probability for an individual is estimated to be correct within 0.24 at the outside with approximately 95 percent confidence, whether the values of the variables were measured at the start of study or whether the changes in the values of some of the variables after 2 weeks of study were used. This method of analysis is general and could be applied to related medical problems. The most important variables measured at the start of the study are age and type of leukemia. The most important variables measured over the first 2 weeks of study are age, type of leukemia, change in performance status, and change in hemorrhage status.

REFERENCE

(*1*) Anderson, R. L., and Bancroft, T. A.: Statistical Theory in Research. New York, McGraw-Hill Co., Inc., 1952.

Basis for Seeking New Types and Structures for Chemotherapeutic Agents[1]

Chairman: DR. HOWARD E. SKIPPER, *Southern Research Institute, Birmingham, Alabama*

Members of Panel:

Dr. Bernard R. Baker, *Stanford Research Institute, Menlo Park, California*
Dr. Ralph Jones, *Jackson Memorial Hospital, Miami, Florida*

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[1] Presented at Conference on Experimental Clinical Cancer Chemotherapy, Washington, D.C., November 11, 1959.

Dr. Skipper: I will begin this panel with a few remarks about purine antagonists.

There are two strategic approaches to the search for new types of structures for cancer chemotherapeutic agents:

Approaches

1. Screening or trial and error
   (*a*) Broad evaluation of synthetics
   (*b*) Evaluation and isolation of natural products (antibiotics, plant products, etc.)
2. Semirational drug design
   (*a*) Knowledge of metabolic difference (neoplastic versus normal)
   (*b*) Concepts of drug action; binding, etc.
   (*c*) Mechanisms of drug resistance
   (*d*) Structure-activity relationships resulting from designed synthesis and quantitative biologic evaluation

The most nonproductive argument one can get into is belaboring the relative merits of screening versus semirational drug design. This question will be settled many years hence—if and when we write the history of the development of drugs which will control or cure the major classes of human cancer.

The screening or trial-and-error approach is criticized because it insults our ego or damages our self-picture. The necessity of screening is a continual reminder that we still do not know much about molecular biology, biochemistry, or pharmacology as far as cancer is concerned, and that you clinicians cannot get much mileage out of a good bedside manner when it comes to neoplastic diseases.

The screening approach to uncover anticancer agents can be no better than the degree of correlation of response between the experimental test system and the human disease. The Cancer Chemotherapy National Service Center uses three animal neoplasms in its primary screen: Sarcoma 180, Adenocarcinoma 755, and Leukemia L1210. These were good as a beginning choice because one or another will pick up amethopterin, 6-mercaptopurine, 6-chloropurine, thioguanine, 5-fluorouracil, 5-fluorodeoxyuridine, urethan, alkylating agents, azaserine, cortisone, actinomycin D, and congeners of certain of these agents, some of which have been useful in man.

The weakness in our present screening system lies in the fact that cancer in man is not (from the chemotherapeutic standpoint) three diseases—it may be three hundred. You all realize that we could not use a single type of human cancer as a screen for predicting the response of all classes of human cancer to drugs. It would be a near biologic miracle if our three animal neoplasms "predicted" all classes of animal or human cancer. Efforts are being made to overcome this weakness in the present screening approach.

Eagle and Foley have found that, if human cancer cells and animal cancer cells are grown side by side in tissue cultures, both respond to the same drugs at almost the same concentrations. This is a very important basic observation.

In our approaches for new types of structures for therapeutic agents, what all of us, who try to design drugs on a semirational basis, want to know more than anything else is a biochemical difference between cancer cells and all vital, normal body cells. Suffice it to say that such knowledge is not yet at hand. Someday the exact site of the biochemical lesion or lesions responsible for neoplasia will be discovered. I imagine that such knowledge would contribute to rational drug design.

There are few classes of human cancer which can be "almost" cured by chemotherapeutic agents. The seemingly complete but temporary clinical remissions seen in acute leukemia after amethopterin or 6-MP treatment, and the response of choriocarcinoma in women after amethopterin are examples. It would appear that development of drug resistance might be the stumbling block to cure or effect long-term control of these types of cancer. Thus, it seemed important to my associates to learn why cells become resistant to a useful antileukemic drug, 6-MP.

It took about 7 years to find what appears to me to be the answer which is so simple that one wonders why it should have taken so long. Briefly, 6-mercaptopurine must be converted to a 6-mercaptopurine ribonucleotide to be cytotoxic. Bacterial or leukemia cells that are sensitive to 6-MP blithely convert 6-MP into its toxic form and kill themselves, whereas 6-MP-resistant bacterial or leukemia cells have lost or are deficient in the specific enzyme that converts 6-MP into its toxic form, hence they are immune to or resistant to this antimetabolite.

This appears to me to be rather important basic knowledge, having direct bearing on the problem of chemotherapy. We and others are trying to exploit this knowledge by synthesis of fraudulent ribonucleotides. This is one way of seeking specific structures for chemotherapeutic agents.

Dr. Baker will discuss drug action and binding.

Dr. Baker: Compared to folic acid, the folic acid antagonists, amethopterin, aminopterin, and Daraprim represent a class of compounds that bind to the enzyme, folic acid reductase, through a stronger basicity of the binding group. The important question has yet to be answered: Why do amethopterin and aminopterin show a selective action on some tumor tissue compared to normal tissue, while Daraprim and its analogues show no selective action? Is the selectivity due to deletion of folic acid degradation enzymes in susceptible tumor tissue, and are these enzymes in normal tissue unable to degrade Daraprim? Is there a selective transport mechanism of the inhibitors? Or does the answer lie in some other as yet unexplored area?

5-Fluorouracil-type compounds probably bind stronger to the enzymes utilizing uracil than uracil derivatives. This binding may be due to the greater acidity of the 3-NH of 5-fluorouracil. There is certainly evidence that the selectivity shown by 5-fluorouracil and its nucleotides can be due to selective detoxification in susceptible tumor tissue.

There is also evidence that thioguanine operates by selective detoxification. 6-Mercaptopurine has not been studied as thoroughly as thioguanine from the selective detoxification viewpoint. Nevertheless, 6-MP probably operates by this mechanism, since thiouric acid is known to be excreted in the urine. The selective detoxification of these purines is probably due to deletion of xanthine oxidase in the susceptible tumor tissue.

Finally, I should like to reiterate that one may be able to take advantage of the deletion hypothesis with alkylating agents. Thus the carrier group may be metabolized differently in different tissues. The toxicity of nitrogen mustards to the host varies over a several-fold range, depending upon the carrier; therefore, if normal tissue can metabolize the carrier group to give a less toxic nitrogen mustard and if susceptible tumor tissue does not have this enzyme, a favorable tissue selectivity will occur.

Dr. Jones will discuss the alkylating agents and selections of alkylating agents for clinical trial.

Dr. Jones: The problems that we face in selecting new structures for clinical trial from this massive Cancer Chemotherapy National Service Center program are many and merit a little consideration. We are making or procuring some 40,000 new materials a year and are testing them against a variety of different animal tumors. We are extracting about 1,000 or more active materials from this group of compounds. As the clinical facilities of this country are at the present time adequate to handle less than 100 compounds a year, there has to be a high degree of selection.

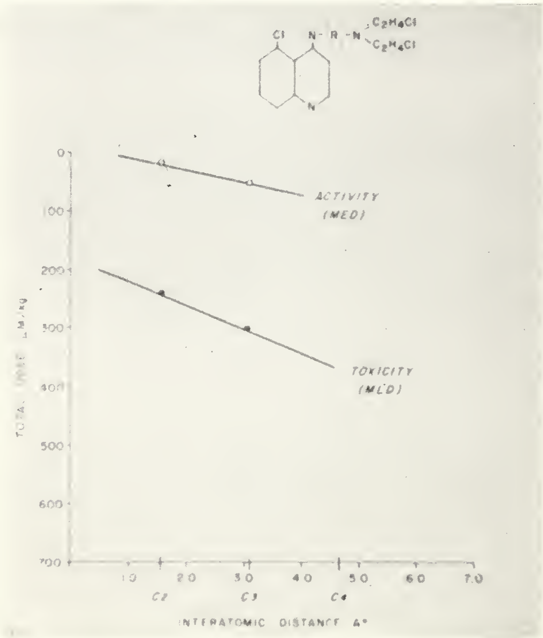
Basically we use a twofold approach: (1) We search for new leads, using many different approaches, trying to find novel structures not known to have any effect on animal cancer. When this occurs and such structures are demonstrated to have a significant therapeutic effect against any animal tumor system and this effect can be demonstrated reproducibly, we have no option but to test that material by clinical trial. This is true because at present we cannot rate our animal systems in terms of their ability to predict. We cannot discard a drug, with a limited therapeutic effect against only one type of tumor, because we are unable to say that it will not have any effect in man.

(2) This approach lies in exploiting existing leads. The chemists are modifying systematically the structure of a variety of different materials that are known to have some chemotherapeutic activity against human disease. These have been classified previously. But one thing is certain: Of the agents that have therapeutic effect against human cancer, nearly all are agents in which the effect can be demonstrated only in amounts very close to the maximum-tolerated dose in man, *i.e.*, there is a small

margin of safety or a very small chemotherapeutic index, in pharmacological terms.

When the chemists systematically modify the structure of a compound of known structure, they immediately deluge us with a number of materials that we cannot begin to test clinically. In doing this the chemist is seeking to improve the chemotherapeutic efficiency of the compound: the margin of safety or the chemotherapeutic index. This can be accomplished in several ways: The inherent toxicity to the host can be reduced without a comparable loss of chemotherapeutic activity; the inherent chemotherapeutic activity against the tumor can be increased without an increase in the host toxicity; and most helpfully, these two can be dissociated so that sequential structural analogues will result in a reduction in host toxicity and an increase in chemotherapeutic activity.

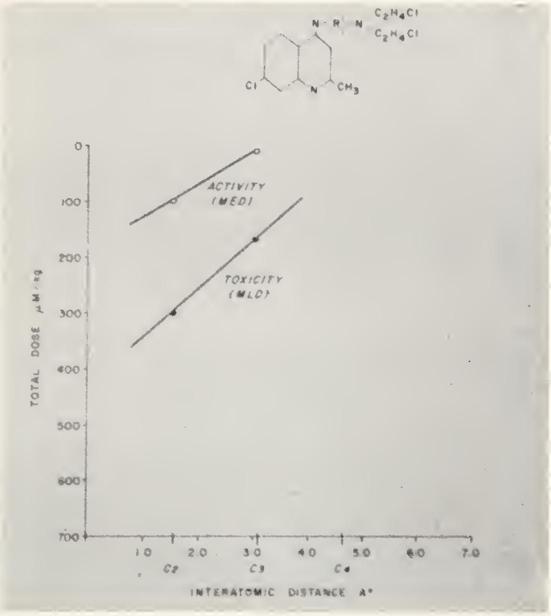
A series of compounds that has been prepared by a cooperative effort of investigators in various institutions is shown in text-figure 1. The synthesis was done principally by Dr. Peck and his colleagues at Lankenau Institute, by Dr. Price at the University of Pennsylvania, and by Dr. Elderfield at the University of Michigan. The nitrogen mustard was attached to the quinoline ring with nuclear modifications, and the length of the alkyl side chain between the two nitrogens was modified so that it had either 2 or 3 carbons.



TEXT-FIGURE 1.—The effect of varying the side chain on biological activity.

The minimum effective dose, in μmoles per kg. of total dose, and the minimum lethal dose are plotted in the same terms. As the number of carbons was increased, toxicity increased and chemotherapeutic activity increased in a parallel fashion, with the net result of no gain.

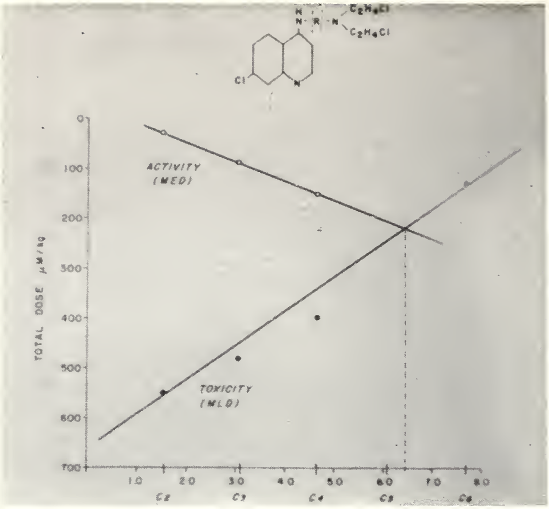
Text-figure 2 shows the structural modification of another compound, a 5-chloro derivative, in which the same alkyl moiety was modified, and as the length of the side chain was increased there was a slight change in chemotherapeutic activity: a reduction and a parallel reduction in toxicity.



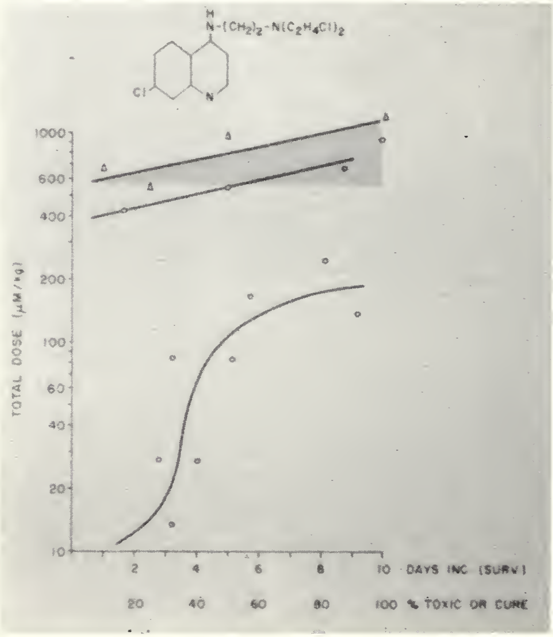
TEXT-FIGURE 2.—Structural modification of a 5-chloro derivative.

Text-figure 3 shows a series of compounds in this series in which the same structural modification was made in the 7-chloro series. To start at the 5 carbon side chain, about 150 μmoles per kg. were required to demonstrate therapeutic activity, and the drug killed at a level of about 400 μmoles per kg. When the side chain was reduced to 3 carbons, therapeutic activity was demonstrable at 75 μmoles per kg., and lethality occurred at about 490 μmoles per kg. When the side chain was reduced to 2 carbons, chemotherapeutic activity was demonstrable at about 10 μmoles per kg., and lethality did not occur until 550 μmoles per kg. This is the type of dissociation between chemotherapeutic activity and toxicity that one seeks in a synthetic program of this type.

The data presented in text-figure 4 show the 2 carbon member, ethylchloroquine mustard, and its effect on the Dunning leukemia. The controls survived an average of about 15 days, and as the dose was increased, mg. per kg. per day or μmoles per kg. per day, there was an increase in survival time of 4 days over the controls, then an occasional animal was cured and curative activity occurred before toxicity appeared, and then toxicity appeared with higher doses.

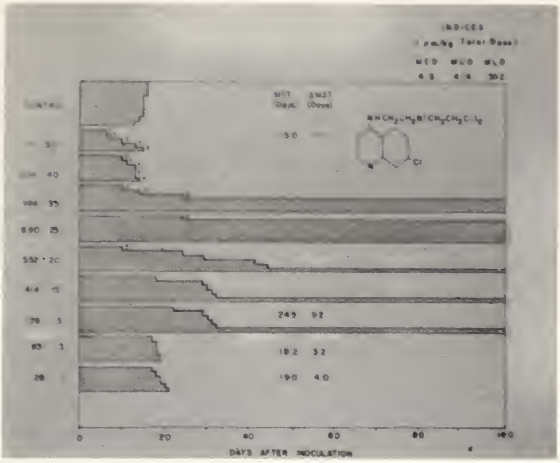


TEXT-FIGURE 3.—Structural modification of a 7-chloro derivative.



TEXT-FIGURE 4.—The effect of a 2 carbon member, ethylchloroquine mustard, given subcutaneously in oil 12×, on the Dunning leukemia.

The chemotherapeutic indexes of 7cl-4 aminoethylquinoline mustard are shown in text-figure 5. The values are plotted on semilog paper. This is the increase in days in the mean survival as the dose is increased, percent of animals cured, and percent of animals killed by the drug. This is the objective of the type of research I have been describing. The fact that this can be done is encouraging.



TEXT-FIGURE 5.—The effect of 7cl-4 aminoethylquinoline mustard.

If, however, we are to test these materials in man we must have animal tumor systems that will select quantitatively among these chemicals so that, when the deluge of active compounds hits us, we can say that a given material in a particular animal tumor system is superior to the previous material and should be selected over it. This can now be done by quantitative pharmacological approaches, in which a number of different animal tumor systems is used. Some of these were mentioned by Dr. Stock. Dr. Goldin's work with the L1210 leukemia is a solid example of such a system. Dr. Schmidt has demonstrated this with the Walker carcinosarcoma 256, and others are doing the same thing. It is certain that in animal tumor systems we can now provide the quantitative information that is required to select among compounds.

The next logical step in selecting agents for clinical trial should be based on data which accurately correlate tumor response in the animal screen with responsive human tumors. At the present time, however, despite these fine animal data, selection for clinical trial must necessarily be based largely on the empirical method and the hunch approach. The reason is that today we have no single animal tumor system providing good quantitative correlation of tumor responsiveness and host toxicity between animal and man.

In my opinion, this is the great objective of the national program at the present time. We must base our selection of agents for clinical trial on one single objective. Unless we obtain reliable, quantitative information in man, sufficient to enable us to interpret the meaning of the various screening systems now in use, we cannot choose intelligently materials that should go into clinical trial. I feel obligated to apologize, for my clinical colleagues and myself, to the basic scientists who have done this very fine work, because we have not yet provided this information. However, our area is difficult and there are reasons why the data are not available. The basis for selection of agents for clinical trial in this program in the future must, in my opinion, be a prime objective. We must learn whether or not we can obtain quantitative data that will correlate with animal tumor systems now in use so that we can predict from the animal which agent should be tested in man.

Dr. Baker: The first question asked of me was to name the tumors that have a high concentration of xanthine oxidase. I inferred that some tumors had a concentration of xanthine oxidase approaching that of some normal tissue. Greenstein's *Biochemistry of Cancer*, 1954, p. 400 lists this data.

The next question is really a comment and a good one: Would it not be standing on firmer experimental ground to refer to the so-called "deletion hypothesis" as a quantitative biochemical difference rather than implying an absolute enzyme lack as possibly responsible for the neoplastic process? I agree that the statement is closer to the experimental facts. I do not know of any instance in which there is an absolute lack of xanthine oxidase. Dr. Skipper, your group has been working on this enzyme system. Do you know if there is an absolute lack of xanthine oxidase?

Dr. Skipper: Dr. de Lamirande in Canada has reported a complete lack of xanthine oxidase in the Novikoff hepatoma. In our laboratory, we find a small amount of xanthine oxidase in this tumor. It is not an absolute lack, but a much lower level of xanthine oxidase activity.

Dr. Baker: When you say there is a small amount, what is the percentage as compared to normal liver cells?

Dr. Skipper: About 5 to 10 percent.

Dr. Baker: Are there any drugs which will increase the growth rate of solid tumors? I think the Service Center has had drugs that do this.

Dr. Skipper: Our group has been looking for this sort of thing for many years, but I do not know of any material that will reproducibly and significantly stimulate the growth of a tumor *in vivo*, except hormones in hormone-dependent neoplasms. Dr. Hitchings asked if we have studied kinetin carefully. We have evaluated kinetin, using the regular chemotherapeutic assays, but have never extensively considered it. I think all of us would be tremendously interested to learn of any nonhormonal chemical that would consistently and significantly increase the growth rate of an animal or a human neoplasm.

Professor Bergel has asked if larger molecules, such as oligonucleotides, consisting of changed purines, pyrimidines, or phosphoric acids, have a better chance of being selectively active than smaller molecules. This is a good question, but I do not know that any one can provide the answer, since extensive work has not been done in evaluating oligonucleotides having fraudulent purines or pyrimidines. Several groups are working in this area and I think this is a worthwhile, possibly productive, approach. I have one other question: Is there any information available to indicate that resistance to 6-MP in human leukemia is associated with loss of ability to convert 6-MP to a 6-MP ribonucleotide? I cannot answer this question. I again can assure you that there are two or three groups working on this problem. The ideal way, it seems to me, would be to determine the amount of inosinic acid pyrophosphorylase in the leukemia cells of a patient who had never been treated with 6-MP. After the patient had responded to treatment, 6-MP would be continued until he became resistant. Then the leukemia cells would be tested again to see, on a per cell basis, if the level of the pyrophosphoraylase had gone down significantly. Dr. Davidson at the National Institutes of Health is working on this problem, and we are collaborating with Dr. Burchenal and his group.

Dr. Baker: The question is asked: Is there even a reasonable chance of reducing the reactivity of alkylating agents, *i.e.*, mustards, epoxides, and ethelenimines, so as to permit the specificity of the carrier to express itself? This is an important aspect of the problem. The nitrogen mustards as a class can vary from a very short half life, such as HN2, to a reasonably long half life, such as chlorambucil. The main thing, as I see it, is that the drug given to the animal or the patient must reach the site. To reach the site depends on the solubility of the drug in the blood stream and the transport into the cells, though other transportation mechanisms may be possible. If the compound is transported too slowly, compared to the reactivity of the alkylating group, the compound is not going to reach the tumor intact. Experimentally, if a good carrier were known, the different types of nitrogen mustards, epoxides, and ethelenimines that could be put on that carrier could be studied; in fact, we are studying this now. We have a one-armed mustard derived from 6-amino-D-glucose that shows activity with L1210 leukemia and we are trying to see if we can increase the efficiency of the drug by increasing or decreasing the chemical reactivity of the chloroethylamine group. This may be an approach to the problem of increasing the effectiveness of a given carrier.

Dr. Skipper: Exploitable biochemical differences formerly referred to enzymes essential to the metabolism of cells whose activity differs greatly between normal and tumor cells. Is the general thinking now shifting from this concept to the hypothesis that the important enzymes to look at are the ones which detoxify or toxify the candidate chemotherapeutic agents? Certainly it is important to look for enzymes that are low or absent in neoplastic tissues so that one can attempt to design a drug that perhaps will be detoxified by normal tissue and not by neoplastic tissue. Dr. Arnold Selegman has been studying this for a number of years with his alkylating agents which are blocked in one way or another.

Investigation of Therapy in Solid Tumors [1]

Chairman: **DR. LYNDON E. LEE, Jr.,** *Veterans Administration Central Office, Washington, D.C.*

Members of Panel:

Dr. Anthony Curreri, *University of Wisconsin, Madison, Wisconsin*
Dr. Bruce Shnider, *District of Columbia General Hospital, Washington, D.C.*
Dr. Jacob Colsky, *Jackson Memorial Hospital, Miami, Florida*
Dr. Ulfar Jonsson, *Jackson Memorial Hospital, Miami, Florida*
Dr. Henry Close, *Veterans Administration Hospital, Philadelphia, Pennsylvania*

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[1] Presented at Conference on Experimental Clinical Cancer Chemotherapy, Washington, D.C., November 11, 1959.

Dr. Lee: We as a panel would like to protest this designation of "solid tumors." It is an unfortunately loose term covering almost all neoplasms; I have from good authority that there are in the *International Classification of Diseases* some 80 or so tumors, most of them solid. It is a patently untrue designation if we imply that the life histories of all these so-called "solid tumors" are similar, with the same diagnostic characteristics, the same responses to therapy, and the same prognosis. None of these things are true.

It is a dangerous implication insofar as analogies may be drawn, interpolating from response or lack of it in one tumor to others with which we may be dealing. We therefore pose the issue that "solid tumors" is not a good term.

Dr. Ravdin has outlined some of the accomplishments of this program. May I be more specific and tell you what some of us feel are its accomplishments outside the factors of chemical treatment of cancer, from the standpoint of clinicians. The program has made possible the organization of groups of investigators with diverse talents and interests, collaborating on a general problem. It has evolved a more universal understanding of language, definitions, and mechanisms in many types of malignant new growths. It has instructed a large and growing number of clinicians and basic science workers in this improved communication. The country's most skilled and experienced clinicians have evolved the best regimens for therapy of patients with specific cancers. Obviously it has fostered the application of these "best" regimens.

In the selection of patients for the studies, the program has improved attention to them and to others, and it has brought to the attention of both clinic and laboratory workers the necessity for biometrics as a science in our work judgments and assays of results, as well as in evaluating the causes of those results.

Also, and I believe this may be a somewhat neglected area, there is initiated through the End Results Program of the Cancer Chemotherapy National Service Center and its cooperating registries a far clearer picture of cancer as a problem affecting our population. It has brought us a more thorough understanding of what results we may expect from therapy in these specific cancer types.

Last, and by no means least, it has brought to light the true morbidity and mortality in certain of our therapeutic procedures, particularly surgery.

In short, the program may be credited with a tremendous improvement in the care of cancer patients in the United States, particularly the patients with advanced cancer. We have an enhancement of our clinical knowledge and its relation to basic science, and we have an improved communication among our physicians, especially wide communication as to advances in therapy.

Now the charge to this panel: "On the knowledge of where have we been; where are we now; and how did we get here?" to consider

"What do we know; where should we go; and how should we get there?" This then, is the charge, and to initiate some discussion (possibly even to generate a little heat) I shall pose the panel initially with the statement that "solid tumors" is unclear, untrue, and a dangerous designation, and that "cancer chemotherapy alone never cured any solid tumor."

Dr. Curreri: That cancer chemotherapy alone never cured a solid tumor is a dangerous statement to make. You would be on firmer ground if you would say that, up to the present, cancer chemotherapy has controlled lesions. There is some evidence that chemotherapy in choriocarcinoma may produce cures, and we still do not know what some agents may do in a few isolated instances. It would be much safer for the public at large and all of us to think in terms of control up to the present, rather than talk about cures. No one can talk about cures until 15 years elapse after we initiate the compound for therapeutic trials.

Dr. Shnider: Should we say we have not to date cured any solid tumors or should we dilute that statement and say that we have achieved some measure of control, but we do not know whether we have achieved any cure? I think we are playing with terms. We have not to date really demonstrated a cure of cancer with chemotherapeutic agents, though we have demonstrated possible promise thereof with choricarcinomas, which Dr. Curreri has suggested. It will take time to tell whether or not we have achieved cures, but we do not appear close to our goal by any means and we must constantly keep searching for better drugs, better criteria for assaying our results, and better methods for choosing patients in whom we are going to assay our clinical investigations.

Dr. Colsky: Certainly there are problems in investigation of drugs and the treatment of solid tumors. I use the words "solid tumors" in the sense that they have been used in the past, realizing that each tumor in itself is an entity, and one cannot say that failure in treatment of one type of solid tumor is sufficient to state that another type of solid tumor will fail to respond to that therapy. This, of course, enlarges the amount of work that any one particular agent must have done on it to state that it either has no or some positive effect.

The problem of investigating drugs is large, and the clinical material, though there appears to be much available, is not as plentiful as one might think. Patients can be separated into two categories: those who have objective tumors that one can look at and measure and for which it can be stated that this drug has caused regression, and patients who have no measurable tumors and in whom symptoms are the major situations in which one can measure drug effect.

To study a drug, one has to know something about its pharmacology, and the so-called phase-1 or initial human pharmacology studies are badly needed long before one goes into the investigation of treatment of tumors. One can use patients with measurable tumors and patients without measurable tumors for situations like this. Once initial human pharmacology has been established, one has to go on to more objective criteria for measurements.

One of the things that has plagued me is the insistence of some individuals upon controls for every study. Some means could be found by collaboration of various groups in this national program of establishing standard controls for each tumor, even if each group contributed 5, 10, or 15 patients treated in a standard way. Methods would be agreed upon after conference and according to what the statisticians might tell us is a just number to draw conclusions from. For example, 100 patients with carcinoma of the colon might have been studied and their courses would appear to define the natural history of colon cancer. This could then be used as a historical control, and there would not be the need constantly to utilize controls, which seems a waste of patient material.

Dr. Jonsson: I agree with Dr. Colsky. The internal standards that we have been trying to build into many of our studies should not be necessary. If we knew more accurately the natural histories of the individual different tumors, we could use those as a basic reference or we could have our group treated by a standard method and thereby increase our output twofold by not carrying standard groups in every study.

I also feel that the groups as a whole have been somewhat timid about studying new compounds as they come through the screen. Our biggest problem is that there is a tremendous number of drugs coming through that show some effect in the screen but have not even been touched in a human being.

Dr. Close: Before I go on with the theme with which I partially agree, we should emphasize that there are other benefits being derived from cooperative studies. Initially when we began we had 11 hospitals with 11 different investigators. We had to get on some common ground as far as criteria for selection of patients and evaluation of tumor response, and it took us at least a year to accomplish some agreement in thinking with other investigators. This is the initial benefit that all groups derive from working together and, as was suggested, this could be carried further in trying to develop common areas of agreement, not only among group members but among the various groups. It is only in this way that we can compare our results with other groups.

We probably all have different ideas about evaluating tumor response and this might be the time to state what our particular group does. We work in a rather narrow channel for evaluation. We are in the Veterans Administration solid tumor group, as Dr. Lee stated. Our solid tumors, exclusive of lymphomas, consist of bronchogenic carcinoma and melanomas. Since melanomas are too infrequent and so far have not responded to anything, we will not discuss them. In bronchogenic carcinoma, the first criterion for admission of a case to the study is that we have a measurable lesion. It does not mean that we are measuring areas of questionable tumor, areas of atelectasis, or areas of pneumonitis. We want to feet that, as far as is possible, we are actually measuring tumor area, and then we go on to compare nitrogen mustard with various new drugs for actual tumor response.

I would like to comment on this response of measurable tumors. Good measurable lung lesions, and I suppose other tumors also, are few and far between. I would guess that only about 10 percent of the nonoperative cases of lung carcinoma would have measurable lesions. We recently reviewed 73 of our cases that had nitrogen mustard and, of this group, we found only 5 that meet our criteria for a measurable response. True, these criteria were fairly rigid. We wanted to have a 20 percent or more decrease in the linear measurement and a 40 percent decrease in the product of 2 measurements, not considering any lesion below 1.5 by 1.5 cm.

I do not know what the other members use as their criteria but we work in a very selective field. We felt that, with such a low level of response by the criteria that we were using, this might be an ideal group of cases in which to do human screening of drugs. One would not need many negative responses to eliminate a drug as unsuitable in treatment of bronchogenic carcinoma.

Dr. Lee: Dr. Curreri, what tumors are you working with in your cooperative studies, and what criteria may be used not alone in those, but in general?

Dr. Curreri: The cooperative studies of the surgical group will be discussed by the next panel and I therefore will utilize my time to discuss (1) our criteria for improvement and (2) patient selection. I believe the parameters used to determine improvement are so important that, because of confusion and disagreement, the Cancer Chemotherapy National Service Center should make a real effort to bring a group together to establish definite standards for reporting to them. This would promote a standard for comparison. Such basic standards would not deny individual investigators the expansion or inclusion of additional criteria.

Very little can be determined regarding therapeutic claims made in the literature. Some investigators accept subjective or symptomatic relief as an index of improvement, while others use objective improvement. It is most important to appreciate that total improvement is our objective, which includes symptomatic and objective improvement as well as improvement in physical and performance status. During the past 2 years our criteria for improvement have been (1) objective measurable improvement in the lesion, (2) symptomatic improvement, (3) a leveling off or reversal in the weight curve, (4) improvement in patients' performance status, and (5) maintenance of all these improvements for a minimum of 2 months. Such criteria would exclude many reports of improvement from 3 to 8 weeks. Certain tumors such as carcinoma of the stomach might require improvement for at least 3 months because many patients with these lesions have improved on good strict medical management for periods of 6 or 8 weeks. For these reasons I hope the participants in the Cancer Chemotherapy National Service Center program will agree on the basic reporting to the center to permit a proper evaluation of the many compounds that may become available.

The question of patient selection is also important. We have been interested in the field of the antimetabolites with 5-FU and 5-FUDR, as well as the combination of azaserine and thioguanine. It has been our philosophy to push these compounds to the point of toxicity because tumor regression was seen at this level and also because this level might indicate a therapeutic level in the tumor tissue. Others have disagreed and believe it is not proper to go above a subtoxic level. It would be reasonable therefore for investigators to pursue both approaches consistently. If this were done, both methods could be compared.

If either of these two regimens is utilized, one should be careful in patient selection if he is to avoid serious toxicity, complications, and even death. We have divided our patients into two groups: (1) average-risk and (2) poor-risk patient. Terminal cases are never included in these studies.

The average-risk patient can receive the established basic dosage schedule as approaching toxicity. However, if the poor-risk patient is unable to tolerate the basic dose, he is given a trial dose, about two thirds of that given to the average patient. The patient is observed during the month and, depending upon the toxic response, the next month's dosage schedule is gauged.

A poor-risk patient is one who has had (1) excessive protein loss as shown by marked cachexia or tremendous pleural or peritoneal effusions, (2) extensive osseous metastases which markedly reduce the total bone marrow mass and thereby develop extreme depression with small amounts, (3) extensive irradiation to the pelvis as occurs with carcinoma of the cervix, for example, where the entire pelvis, the lower vertebra, and the upper femora, would be affected. These patients also develop profound depression of the hematopoietic system, and (4) patients with tremendous liver enlargement and metastases associated with jaundice. These patients tolerate antimetabolites poorly.

Thus, if patients were carefully screened, one could approach toxic levels in administering these compounds and avoid serious consequences.

Dr. Lee: Dr. Colsky and Dr. Jonsson, will you discuss the criteria that we should utilize, particularly from the standpoint that we should offer better information to our chemists to synthesize more carefully and more accurately some promising drugs in the tumors we are discussing.

Dr. Colsky: One has to observe both the symptomatic and the objective status and response of the patient. I am somewhat prone to look askance at symptomatic improvement. In 1951 we were studying some antifoles in solid tumors. It became evident to us as we treated some of these apparently terminal patients, who had been bedridden and starving for weeks, that when we gave them relatively small doses of experimental drugs, which could not have had any real therapeutic effect, many began to feel much better. They started to eat, in fact some gained weight, many began to get out of bed, and they seemed to be considerably improved. We did not believe for one moment that this was drug effect for, as the drug was continued, the results were quite obvious. The patients lost their feeling of well-being, not due to drug toxicity but because their euphoria had disappeared, and they returned to their former state.

This does not imply that we should discard completely symptomatic improvement as a measure of response. However, it is quite obvious that if one were to have a total objective response, with disappearance of all the tumors in the patient who is still gradually dying, this would not be the type of response we would seek.

I feel that symptomatic and objective improvements are really inseparable. I think we are seeking two things: (1) a cure for cancer, which is a rather long-range and optimistic approach; (2) drugs which give a measurable effect upon the disease so that (*a*) we can attempt to learn more about these drugs as far as their metabolic or other activities are concerned, (*b*) we might indicate to the chemist that these are effective drugs, although they may be relatively poor in their effect, with objective remission and symptomatic remission lasting only a short period. There is hope if uniformly they cause more regression of tumors than one sees during natural fluctuations in the course of the disease. This is sufficient a lead for the chemist to attempt to make other drugs along the same lines.

After a sufficient number of analogues have been studied, one will have to determine when to stop exploring this particular avenue for more active drugs. As far as symptomatic improvement is concerned, there have been many attempts to measure this by assigning certain values to each function of the individual. Although this is something that must be done, I personally feel that it should not hold the major role, but that we must have objective evidence of improvement of the patient and the tumor.

Dr. Jonsson: Subjective improvement without any regression in objective tumor probably in most cases will prove completely useless criteria. However, in the leukemias and lymphomas, for example, certain defined percentages are of use in objective measurements, as with leukocytes.

In the "solid tumors," on the other hand, these criteria are of much less value. And in the absence of objective regression, I think subjective improvement should be ignored.

Dr. Shnider: The problem of different criteria used by different individuals is a real one for groups in solid-tumor therapy. There has to be some common meeting ground. Whether this is to be resolved by representatives of all the groups or by some commission, criteria of response have to be very carefully evaluated.

Initially when we set up the Eastern solid tumor chemotherapy group we thought, possibly very naively, that we could go by objective regression of tumor masses alone, tumor masses that we could feel and measure, or tumor masses that we could see on X ray and to which we could apply calipers. We would record other information, but our primary evaluation of response was on this so-called objective measurable disease.

Now it has become evident to us in continually reassessing our criteria for response that this is not adequate. What is adequate I do not know. Much deliberation is necessary before we can say. After all, when we talk about objective measurable disease we have rather inaccurate criteria with the few exceptions of the tumors that one can see and directly measure. X-ray evidence of what we think is tumor mass, in spite of the fact that we put calipers on it, does not necessarily mean that we are assaying true objective measurable disease.

How much influence the improvement in a patient's performance status should have is another factor that merits further consideration.

Now we can go to selection of patients. Are we all selecting the same type of patients for our studies, so that when we talk about effective drugs we are talking about as nearly similar situations as possible?

Do we choose the same type of patient for a phase-1 study, phase-2 study, or phase-3 study? And what are these phase-1, phase-2, and phase-3 studies? These are other problems in the field of solid-tumor investigation. Do we choose the same type of patient for a phase-3 study, which most of us believe to be the final comparative evaluation, as for the initial investigation of the toxicity of a drug in establishment of dosage regimens?

Further, how do we choose the drugs we are going to investigate? We get information from the animal tumor screens that tell us a drug has certain activity, but it has been evident to those of us who are using these drugs in patients that activity in these screens does not necessarily apply to the drug activity as it appears in the solid tumor group of patients.

In other words, are the animal screens giving us information for all the types of patients we are dealing with? I think most of us would honestly answer, "No." This means that we need new types of screens for certain types of tumors. Even supposing that those exist, how do we choose the drugs that we are going to use for investigation?

Dr. Close: In emphasizing that we use measurable lesions perhaps I gave the impression that we ignore the patient's symptoms. We do not at all. The patient's symptomatology, weight, temperature, and other things are recorded and they are analyzed in the final data so we do not ignore them, but our main consideration is the change of measurable lesions. When we get into symptomatology in evaluating the patient's subjective response, this brings up another problem, which is introducing the "best medical care" group into more of our studies.

It has been suggested that perhaps we can get the natural history of the disease from the End Results Program. I do not think this is possible because many of those people have had a variety of treatment, whereas if we can introduce the "best medical care" group into more of our studies we will have a better way of evaluating just what is "best medical care"; *i.e.*, what will bringing a patient to a hospital, giving him food, blood, and other supportive measures do for his general improvement?

Dr. Lee: Do we know whether there is a difference in well-being or results between the patient who gets general supportive care and nothing else, as compared with the patient who has disseminated malignancy and goes from one drug to another before he dies?

Dr. Close: No, we do not know that, and I often wonder whether we should not have people in these studies that are just receiving "best medical care" and not drugs.

Dr. Lee: Do you feel that it would be morally correct to treat all patients with now untried agents when there is a known effective agent? This would happen, of course, if you drop positive controls.

Dr. Colsky: If there is a known really effective agent (and the definition of "effective" would have to be made clear to me before I could answer that question), there would be no reason at all for not treating an untreated patient with this drug. Now if it were totally effective, there would be no need to seek further. Of course, if it were no more effective than the drugs we have today, I think we can all predict that it would be necessary to treat this patient further. I believe patient care must come first, but when there are groups of tumors in which there are no known effective agents (and we know that there are plenty of them that have no known effective methods of treatment), these people can be given the best possible medical care without any detriment to them whatsoever. In fact, at times on "best medical care" they suffer less detriment than when getting some drugs we have available.

Dr. Curreri: May I discuss a question which was brought up and not fully answered: How can we as clinicians help the chemist? Since the clinicians and the chemists have much in common and have much to learn from one another, they should complement one another by working as a team. For example, if tagged material is used, the surgeon can provide clinical information and can assist in the metabolic studies by obtaining blocks of tissue at the time of surgery so that the chemist may determine not only the radioactivity but also the amount and metabolic state of the therapeutic agent. Such studies would provide the chemist with leads in developing analogues or other more effective compounds. A closer working relationship among the chemists, the clinician, and surgeon is needed if we are to achieve our goal efficiently.

Dr. Colsky: The question is asked: At the current point in the solid tumor program (1) how many compounds have caused reproducible objective improvement, and (2) which are they? I have not counted the number of compounds, but I think we could state that the large group of compounds could be broken down into the hormones, the alkylating agents, and the antimetabolites. As to the second part of the question, in my experience and I think in the experience of most people, a certain percentage of breast carcinomas will definitely regress, measurably and without any doubt, when treated with both androgen and estrogen variations, depending on the age of the patient.

As for the alkylating agents, if we take breast carcinoma and ovarian carcinoma, there is general agreement that a definite percentage of regression occurs. I personally have been able to confirm this and I know others have reported it, so I would feel this is reproducible.

Finally, in the field of the antimetabolite there are at least two experiences in which there was an approximately similar regression rate. None of these approached cures, obviously. There probably are other drugs which produce so-called reproducible results in solid tumors. I do not know of them and I have not broken this down into each individual drug within each of the large groups.

As to the question: Which as yet untried drugs should be studied next? I feel that we do not know where we are going at the moment and which drugs should be studied next. If we had a better screening program, if we had means of saying that this response in animals means there is going to be a response in humans, we would be well on the road to saying which untried drug should be studied next. We do not have that. Therefore, we have to study as many drugs as we possibly can. The animal screen to date, as far as I personally am concerned and poor as it is, is the best we have. Along these lines I wonder if something like this might not be considered: Since we feel most people in these various groups are qualified and we can trust their results, possibly each group should be given drugs, or at least be given data on all the drugs, to select 2 or 3 that they want to study particularly. Maybe 3 or more groups should study a single compound and report results, or study 2 or 3 compounds and report results, while other groups might be studying other compounds. At the end of a certain time all results would be made available. If everybody found that the compounds studied were completely negative, it would be up to individual investigators to indicate trust in the results of these 3 other groups. If the results were accepted as competent, other investigators need not be interested in this further. If one or more investigators wished to gain further information or personal experience to test previous reports or other avenues of approach they might proceed without involving the national program.

If any investigator came up with information other than what had been presented, this could then be fed back into the national program and be made available for further analysis. In this way, I believe that more of the so-called phase-1 or initial human pharmacology studies could be made. This would then make drugs available for the so-called phase-2 study of tumor response. If one saw significant tumor response (whether it is symptomatic or objective or both, although I think both are necessary) then one could say, "Now we should compare which is the better dosage regimen; is this a better drug than something else that we are using?" I think the so-called phase-3 ought to wait until we have demonstrated effectiveness of a given agent.

Dr. Close: I stated that 5 of 73 patients with bronchogenic carcinoma had a measurable improvement with nitrogen mustard. Now the question is asked, Is it necessary to continue comparative studies to obtain information on new compounds? This has certainly occurred to us and we are thinking along these lines. Perhaps it is not necessary to continue a comparative study. For example, when we receive a new compound that has some activity in the animal screen, we could initially do sufficient phase-1 studies to arrive at dosage schedules and toxicity evaluation. Then we could treat with this new agent these cases with measurable bronchogenic carcinomas. At one time we discussed this with the biostatisticians. They felt that if we had 16 entirely negative responses we would have very little chance of missing a compound that was active in bronchogenic carcinoma. If we had 1 response out of 16, we would add 5 more cases. If we had 2 responses, we would add 10 more cases and so on. This is what I was referring to when I said perhaps we could utilize this material to better advantage and use it for screening tumor-active compounds in human tumors.

Dr. Curreri: The question is asked: Does any member of the panel use a drug which he regards as hopeful or of proved value in the treatment of so-called solid tumors? 5-Fluorouracil and its derivative, 5-FUDR, have produced definite tumor regression and patient improvement and have met the criteria for improvement we alluded to previously. The lesions responding were breast, colon, carcinoma of the ovary, primary hepatoma, and, to a certain extent, carcinoma of the cervix.

Other investigators working with these compounds have reported benefit in other lesions. Based on our criteria, no benefit was observed in carcinoma of the lung, carcinoma of the stomach, malignant melanoma, and hypernephroma.

The combination of azaserine and 6-thioguanine demonstrated a very definite anticancer effect upon breast lesions. Unfortunately, our studies have not been completed because, after 5 months of drug administration to the point of toxicity, the depressed bone marrow did not return to normal and any attempt to continue therapy produced a sharp and significant drop in the white blood count. As a result of this, we are returning to the dosage schedule originally suggested by Dr. Al LePage. In animal experimentation he demonstrated that small doses of the two compounds would produce potentiation; therefore toxic levels were unnecessary and hematopoietic depression might be avoided. We are now ready to initiate study, but the supply of thioguanine has been exhausted.

The question is asked: What is the difference in percentage of remission with 5-FU between poor-risk and average-risk patients? Ordinarily, we try not to use 5-FU in patients who have a very large liver and jaundice, but we are sometimes pressured into using it. The jaundice has disappeared, the liver has markedly regressed, the liver-function studies have improved, but the patient goes on to die. These patients have such extensive carcinoma that they are unable to respond.

We have, however, seen definite improvement in a number of patients with extensive osteolytic lesions, when we started with a small dose and worked up toward an average dose. As improvements are noted in the osteolytic lesions, the patient tolerates a larger amount of the compound. The same can be said of cachectic patients who, as they improve nutritionally, can tolerate larger doses.

Dr. Close: I would like to mention something about our experience with Cytoxan or cyclophosphamide. We are very early in our work with this. At this stage we feel that it is probably going to be as good as nitrogen mustard in bronchogenic carcinoma, if not a little better. After the initial treatment the patient can be kept on a long-term oral maintenance dose. Although our group is not doing this routinely at our hospital as yet, we are treating a few patients and we believe we are keeping the tumor under some suppression. This gives a little advantage over nitrogen mustard which cannot be given in this same quantity.

Dr. Lee: The panel has agreed that there should be a central information exchange and feels that the Cancer Chemotherapy National Service Center is the logical area from which this should stem.

We feel there should be cooperative groups which gain further agreement on measurement of drug effects in specific cancers. Perhaps delegates from each of the groups working with specific cancer sites and disease types might repeatedly meet to evolve and polish some criteria such as these.

We have asked, "What do we seek?" and we have answered, "Obviously a cancer cure." Being realists, we have admitted that there is no satisfactory cure or cures at hand, so that we then seek improvement in our patients while we examine additional leads for development of cures, and, if cures are found, we seek means of applying these. We believe this program is the means by which this is best done.

Screening methods, we have agreed, must be improved, with particular attention to correlations with human cancers, which as yet have failed to yield in satisfactory measure to chemotherapy and/or to other methods of treatment. We have said that this program probably offers the best possible development of this type of treatment, and that we must develop more screens in human beings.

We have also said that more certain knowledge is necessary of the life history of specific cancers, both treated and untreated. This is essential for a more accurate assay of the effects of our therapy, drugs, or other. We feel that the End Results Program in the Cancer Chemotherapy National Service Center appears the most ready and reliable means of supplying these data from knowledge at hand, and we urge its greater incorporation in the program with dissemination of these findings to all who might profitably use the information.

Finally, we must avoid the situation of seeking too closely an explanation for good results before gratefully accepting measures which give us these results and applying them to patients.

Investigation of Therapy of Lung Carcinoma[1]

Chairman: DR. ANTHONY CURRERI, *University of Wisconsin, Madison, Wisconsin*

Members of Panel:

Dr. Thomas C. Chalmers, ***Lemuel Shattuck Hospital, Boston, Massachusetts***
Dr. Julius Wolf, ***Veterans Administration Hospital, Bronx, New York***
Dr. Leroy Hyde, ***Veterans Administration Hospital, Long Beach, California***
Dr. Felix Hughes, ***Veterans Administration Hospital, Memphis, Tennessee***

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[1] Presented at Conference on Experimental Clinical Cancer Chemotherapy, Washington, D.C., November 11, 1959.

Dr. Curreri: Our panel is charged with discussing chemotherapy in lung carcinoma. I am sure all of us agree there is a need to attack aggressively lung tumors, particularly if one considers the low rate of operability and resectability as well as the limited 2-year survival rate presently reported from pulmonary resection and radiotherapy. The limitation of the present modalities in treating these lesions demands serious consideration of chemotherapy in the control of lung cancer.

Through the Cancer Chemotherapy National Service Center, a number of compounds have been studied and it would be valuable to assess their effectiveness in carcinona of the lung, particularly since they have been used in an adequate number of cases. In the course of our studies numerous questions and problems have developed.

The question was raised, as in the previous panel, whether it was morally proper to deny patients in the control groups one of these compounds. By the same token a reverse stand may be taken, for we do not know if these toxic compounds are palliative until a clinical trial has been completed. Therefore, is one justified in using these toxic compounds indiscriminately?

The problem of methodology always concerns the clinical investigator since the biological behavior of the individual tumor, selection of patients, dosage schedules, and criteria established to determine improvement have a marked influence on the therapeutic results reported. Many of these problems and questions will be answered in the discussions of the participants in this panel.

Table 1 illustrates a comparative study of nitrogen mustard versus thio-TEPA, nitrogen mustard versus X ray, and nitrogen mustard versus nitrogen mustard + X ray. This study was undertaken by the Eastern cooperative group and included cases which had been completed and for the most part evaluated. Much data have been accumulated which should have some significance. The Southeastern group's small series of 14 cases compared Myleran, thio-TEPA, and chlorambucil. The Veterans Administration group had 130 patients in which they compared HN2 with DON against HN2 and Nitromin.

TABLE 1.—Bronchogenic cooperative clinical studies

Group	Clinical study	Number completed	Number evaluated
Eastern	HN2 (0.4 mg./kg.) vs. thio-TEPA (0.8 mg./kg.)	133	133
	HN2 (0.1 mg./kg.) vs. X ray (3000 r)	39	39
	HN2 + X ray vs. X ray alone	53	30
Southeastern	Myleran (6 mg.) vs. thio-TEPA (0.2 mg./kg.) vs. chlorambucil (12 mg.)	14	14
Veterans Administration	HN2 (0.4 mg./kg.) vs. DON (0.2 mg./kg.)	54	54
	HN2 (0.4 mg./kg.) vs. Nitromin (10 mg./kg.)	76	76

Table 2 shows the results of 613 cases of lung cancer studied by the Veterans Administration group, in which they compared androgens, estrogens, and corticoids versus nitrogen mustard in the control of the disease.

Table 2.—Bronchogenic cooperative clinical studies

Group	Hormonal studies	Number completed	Number evaluated
Veterans Administration	HN2 (0.4 mg./kg.) vs. testosterone (300 mg./wk.) vs. cortisone (100 mg./day) vs. progesterone (10 mg./day) vs. diethylstilbestrol (10 mg./day) vs. optimum care	494	486
	Testosterone (300 mg./wk.) vs. Δ^1-testololactone (300 mg./wk.) vs. Halotestin (20 mg./day orally) vs. HN2 (0.4 mg./kg.) vs. radiation vs. optimum care	119	87

The results obtained by the University and the Veterans Administration cooperative study groups when they used nitrogen mustard as an adjuvant to pulmonary resection are shown in table 3. The University group studied a total of 691 cases and the Veterans Administration group, a total of 411 in both the randomization and double-blind studies.

Table 3.—Bronchogenic cooperative clinical studies

Group	Surgical adjuvant	Number completed	Number evaluated
University	Surgery + HN2 (0.4 mg./kg.) vs. surgery	467	207
	Surgery + HN2 (0.3 mg./kg.) vs. surgery + placebo	224	43
Veterans Administration	Surgery + HN2 (0.4 mg./kg.) vs. surgery	247	116
	Surgery + HN2 (0.3 mg./kg.) vs. surgery + placebo	112	18
	Surgery + NIH	53	18

Dr. Chalmers will discuss the problems of methodology.

Dr. Chalmers: How does one evaluate the responses of patients with carcinoma of the lung to various types of therapy during a comparative trial? I believe that this is a most important aspect to work on now, when no dramatically effective drugs are available, and thus obviate the necessity for developing techniques after better agents have materialized.

I should like to report briefly an evaluation of methods for measuring treatment effect carried out as part of the Eastern solid tumor cooperative group's project. Nitrogen mustard and thio-TEPA were compared by the whole group in patients with solid tumors and carcinoma of the lung. At the Shattuck Hospital, we included randomly assigned control patients who were treated with 2-million-volt radiotherapy; Dr. Margarida Dederick and Dr. Martin Levene did most of the work on this study.

We have analyzed the results of the study by three separate methods, with three different results:

(1) The Eastern solid tumor group circulated among the investigators all the pertinent data on patients, *e.g.*, the response of measurable primary and metastatic tumors, white blood counts, and the patients' subjective feelings of improvement. Without knowledge of the treatment given to the patient, the members of the group voted whether they thought he had benefited or not. The results of this method are presented in table 4. There is no doubt from these data that radiotherapy was significantly more effective than chemotherapy in the treatment of these patients.

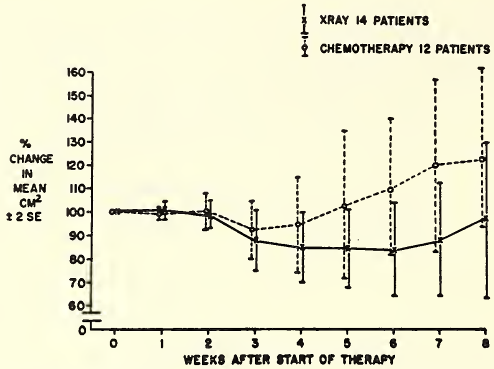
TABLE 4.—Results of poll of investigators in regard to response of each patient to treatment

	Total patients	Reduction in tumor size				Other effects: Major unknown reactions		Other beneficial effects		Total benefit to patient	
		Single tumor		Total tumor mass							
		Yes	No	Yes	No	Yes	No	Yes	No	Yes	No
Radiotherapy	26	14	12	9	17	1	25	6	20	11	15
Chemotherapy	20	4	16	1	19	0	20	1	19	1	19
χ^2		5.36		5.98		—		—		8.1	
P value		<0.05		<0.02		—		—		<0.01	

(2) This evaluation depended upon regular measurement of lung tumor size, in two dimensions, from X rays taken every week. At the end of each patient's study, the dates were disguised and the films randomly distributed for measurement by a doctor who did not know which treatment had been used. Interestingly enough, we found that if we put the films for each patient in proper time sequence there was more often a trend toward a seeming reduction or an increase in size of the tumor than there was when we randomized the films, but we could not help concluding that the latter was a more accurate estimate of the true course of events. In a given patient, the total area of tumor at 2-week intervals was divided by the pretreatment size and averaged for all patients in that treatment group to give a mean ± the standard error, which then represented the change in tumor size that accompanied treatment (text-fig. 1). One of the disadvantages of the use of this technique is that there are different numbers of patients over different lengths of time, because patients drop out of the study for various reasons, largely through death. The number of patients become smaller as time passes, with response to treatment or lack of response, which possibly determines how long a patient contributes to the averages. In spite of this disadvantage, the technique offers a

method for quantitating the mean decrease in tumor mass brought about by each kind of treatment. According to this technique there is a trend in favor of radiotherapy, but it is not statistically significant.

(3) The standard life-table method of recording survival was evaluated. This technique showed no difference between the effects of radiotherapy and chemotherapy.



Text-figure 1.—Comparison of 2 Mev X ray and chemotherapy. Percent change in mean cm.² of measurable lesions. Patients followed for 60 days.

In summary, by the method of evaulation of unbiased votes as to whether there was any decrease in tumor size and whether the patient seemed to feel better, act better, or look better, there was no doubt that 2-million-volt radiotherapy was superior to chemotherapy in the treatment of carcinoma of the lung. In regard to the actual effect of the agents on the tumor, this difference might have been demonstrated if more patients had been included. In terms of the survival time of the patients, there was no difference between the treatments.

The primary conclusion from these data is that several methods of evaluating treatment effect should be built into each study. If one is interested in over-all benefit to the patient, a voting technique is most reliable, but it is woefully imprecise to use qualitative methods to evaluate quantitative data. Survival time is an accurate quantitation of treatment effect, but the disadvantage is that many other treatments may be applied in the terminal phase of the disease. In the final analysis we are interested most in learning whether tumors increase or decrease in size under one particular treatment, and for this we need to work out quantitative methods for handling the data even when the number of patients in the study is constantly decreasing.

I have been assigned the following question: Why choose a minimal-effective dose of X ray, 3,000 r, when it is well established that the optimum dose for epidermoid carcinoma is 6,000 r, given over a period of 6 weeks? I hesitate to answer this without a radiotherapist on the panel. Dr. Levene has been increasing the dose to 4,000 r at the Shattuck Hospital, but we are encountering, albeit rarely, some severe radiation pneumonitis, and I know of no controlled study in which 6,000 r has been established as the optimum dose.

I was shocked to hear one of my colleagues on the previous panel advocate a backward step to eliminate controls and return to the comparison of patients treated with new agents with the previous established series of patients. I think it is time for those of us who believe in controls to take the offensive rather than the defensive, and to point out that the only time we do not need controls is when we know that a new agent is better than the current established treatment, in which case we should not be doing a study. If we do not know whether the new agent will harm the patient more than it helps him, then it is unfair to the patient not to give him an equal chance to be in a control or treatment group. In other words, if we are evaluating a new agent we are morally bound to use controls. Finally from the technical standpoint, the principal reason for controls is not to document the natural history of all the patients with the disease, but rather to document the natural history of the small percentage of patients with that disease who are included in the study.

Dr. Wolf: In February, 1958, fourteen Veterans Administration hospitals began the study of the treatment of patients with inoperable bronchogenic carcinoma. Our protocol is definitive in 4 principal ways:

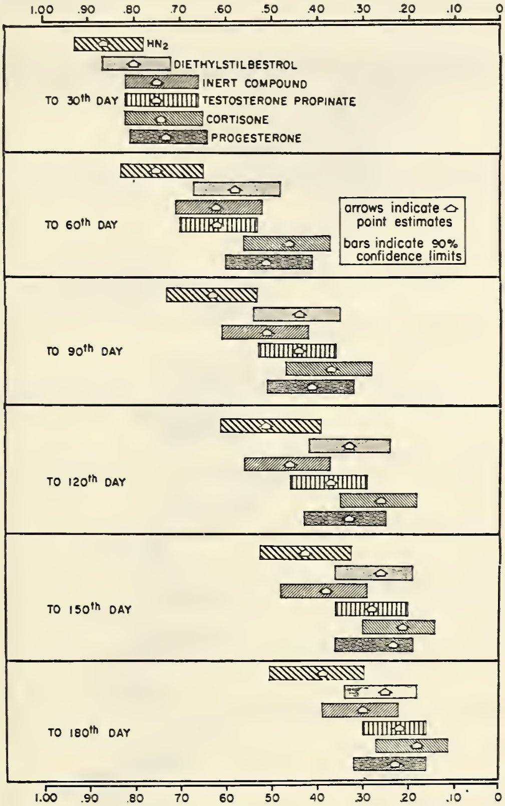
(1) There is no selection of patients; every patient with inoperable bronchogenic carcinoma is accepted for treatment if there is histologic or cytologic proof of the diagnosis. We wanted to avoid the problems involved in matching cases or trying to preselect or predetermine which patients might or might not respond. We hoped the large number of patients available would in the long run provide homogeneity in each treatment group and this is what actually happened.

(2) We felt a control group was needed. While it is true that the natural history of inoperable bronchogenic carcinoma is known to the extent that all such patients eventually die, when they die is not known. This is a problem that varies from hospital to hospital and is dependent upon admission policies and conditions in the community. The statistics from the State of Connecticut, for example, would indicate that half of the patients with inoperable bronchogenic carcinoma died within 3 months from admission to a hospital; on the other hand, patients admitted to the Boston City Hospital died much sooner. We felt that we had to know what the course was in patients admitted to our 14 Veterans Administration hospitals. Also, we thought a control group was important in evaluating possible drug toxicity in a disease which itself can produce all the signs and symptoms ordinarily ascribable to drugs. We have found that when we used a coded placebo investigators ascribed to it toxic reactions. This convinced us that we should use as far as possible the double-blind procedure, fully aware that the best definition of a double-blind study is that of the blind leading the blind.

(3) We were not satisfied with the 3 available methods of measurement: tumor size, performance status, and long-term survival. The defects in each of these have been adequately discussed. In bronchogenic carcinoma we have an unfortunate advantage that many of these patients die within a relatively short time. We thought that the best parameter would be the measurement of the number of patients living to a given date. Thus we could get the study over with in a relatively short time and we would not be involved with the problems of long-term follow up where patients are lost or have received other forms of treatment.

(4) We did not use any of the newer drugs in our first study, but subjected the old drugs that seemed of some value in bronchogenic carcinoma or had been rejected on the basis of inadequate evidence. Nitrogen mustard was chosen because this is the one drug that apparently produces some remission. Cortisone was used because of the many reports that cortisone has a role in the treatment of inoperable bronchogenic carcinoma with its ubiquitous nonspecific effects. On the other hand, animal experiments might seem to indicate that cortisone, by interfering with the immune reaction of the host, may also have a deleterious effect in carcinoma. Then we chose the 3 sex hormones—diethylstilbestrol, progesterone, and testosterone propionate. Several years ago the thought occurred to many that, since bronchogenic carcinoma seems to be more common in men, there may be some relationship with the sex hormones. In small numbers of patients orchiectomy had been performed without any effect. Diethylstilbestrol was also given without too much benefit but testosterone propionate, which should have made these patients worse, actually seemed to make them better. Progesterone was added simply to complete our screen of the sex-hormone prototypes.

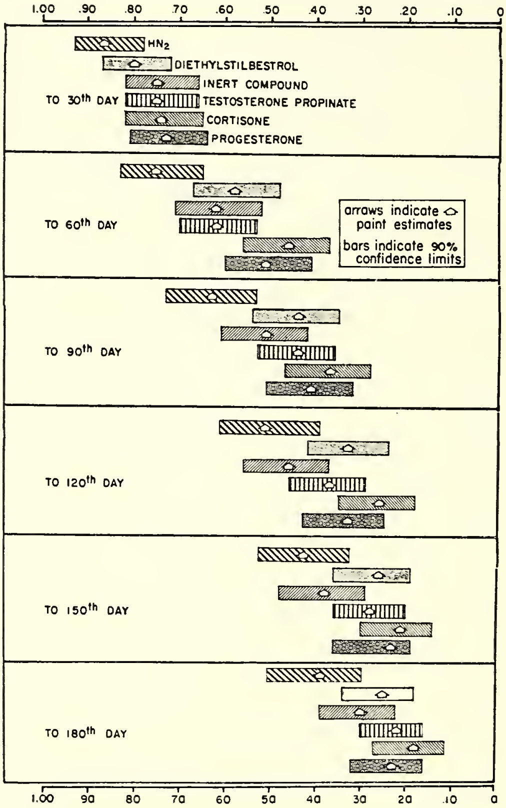
We thus began our study in February, 1958, and carried it on for a year. Five hundred and forty patients, approximately 90 in each of these drug groups, were admitted to the study. Nitrogen mustard was given in a dose of 0.4 mg. per kg. and repeated in 6 weeks. Testosterone propionate, 100 mg., was given 3 times a week for 12 weeks. The 3 other drugs were given orally together with lactose in a double-blind fashion. They were diethylstilbestrol, 10 mg. a day, cortisone, 100 mg. a day, and progesterone in a 2 gm. daily dose. Text-figure 2 shows the results of this study. The arrows point to the average percentage of patients surviving to a given day. The bars represent the limits of the 90 percent confidence intervals. At the 60th day, 75 percent of the patients who had received nitrogen mustard were still living as compared to 62 percent of the patients who had received the inert compound and only 46 percent of the patients who had received cortisone. At the 90th day these figures were 63, 51, and 37 percent respectively. The overlap is such that the difference between the nitrogen mustard and the inert compound is not particularly significant and we cannot say with any assurance that nitrogen mustard has shown any real beneficial effect in these patients. On the other hand, the difference between cortisone and the control group is much more striking. The proportion surviving began to aggregate in all 6 drug groups so that the differences at the 180th day were hardly significant one from the other. Dr. Hyde will present some of the further analyses of our data.



TEXT-FIGURE 2.—Ninety percent confidence interval estimates of the proportion that survive to indicated day.

Dr. Hyde: My remarks are essentially a continuation of those by Dr. Wolf and refer to protocol number one of the Veterans Administration lung cancer cooperative study. As has been indicated, no drug used in this study had any definite or significant therapeutic value as far as prolongation of life is concerned. Several observations were interesting, however.

Text-figure 3 indicates that the first course of nitrogen mustard appeared to exert a slight beneficial effect. The nitrogen mustard bar extends farther to the left than the others, which indicates a slightly higher percentage of patients living. This effect was not noted after the 2d dose of nitrogen mustard. The group (3d line) receiving optimal medical care only has done just as well as the other groups. Cortisone had a definite deleterious effect, most evident during the 2d month of therapy. In the "to 60th-day" group the line extends to the right significantly beyond the others, which indicates fewer patients that were alive. The probability that this difference was the result of sampling variation is less than 2 percent.

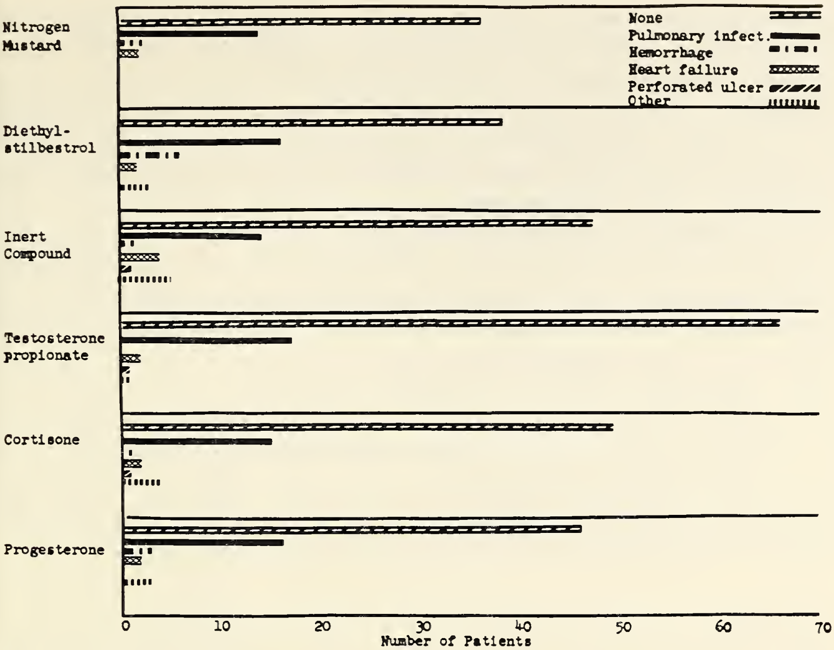


TEXT-FIGURE 3.—Ninety percent confidence interval estimates of the proportion that survive to indicated day. *Top line* of each group indicates the nitrogen mustard bar. Inert compound, *3d line*, represents the group receiving optimal medical care only. The *5th line* in the "to 60th-day" group indicates fewer patients alive.

Attempts were made to discover the reason for the adverse effect of cortisone but none was found clinically (text-fig. 4). The causes of death for each regimen are essentially similar. However, cortisone may affect various immune or enzyme mechanisms which we cannot measure.

One unexpected finding of this study was the higher incidence of terminal massive hemorrhage in patients with keratinizing squamous-cell carcinoma, especially in those patients with this disease who received diethylstilbestrol.

It is expected that the complete data of protocol number one will be published in the not too distant future and will be available for more detailed study. Protocol number two is now in progress and involves the



TEXT-FIGURE 4.—Principal contributory causes of death.

comparison of patients treated with X ray, nitrogen mustard, testosterone, Δ^1-testololactone, Halotestin, and the usual optimal supportive therapy group. After 30 days of therapy 457 patients were reviewed. Although it is much too early for any definite statements, no regimen appears superior to the others. It is hoped that the nonresectable lung cancer chemotherapy Veterans Administration cooperative study will yield valuable information since: (1) All proved nonresectable bronchogenic carcinoma patients are included, thereby eliminating selection of cases; (2) objective measurement, that is the percentage of patients surviving at 30-day intervals is used, thereby eliminating unconscious subjective bias of the clinician, the yearnings and hopes of the patient, and also emphasizing the goal of prolongation of life for the cancer patient; and (3) a control group is included, which avoids the so-called "clinical impressions" of the supposed benefits of some often-used treatments.

Personal experience with about 1,400 cases of bronchogenic carcinoma forces one to conclude that there is a definite small group in which the tumor has a different biologic nature from the other bronchogenic carcinomas. Approximately 3 percent of bronchogenic carcinoma patients, for some unexplained reason, even without therapy, will live much longer than the usual cancer patients, often for 5 years. We secure old chest X-ray films of our patients, whenever possible, and have been surprised to see how often a peripheral lesion has been present for 2, 3, or 4 years. These slow-growing cancers are the ideal cases for surgery and when these lesions are resected, the patients tend to do well, probably because of the absence of blood-vessel invasion. Since we do not know the potential of each cancer, it is best to have all lesions resected, at least until an effective chemotherapeutic agent is found.

Dr. Rigler has reported cases in which X-ray lesions were present as long as 6 and 7 years before resection and histologic diagnosis. I have seen similar cases with X-ray densities present up to 5 years before resection and histologic diagnosis.

We have analyzed the group of patients who live longer than anticipated and have found no reason for this. Where the histologic type has been established in the small number of cases that we have had, it is noted that 80 percent of them are squamous-cell, 10 percent are oat-cell, and 10 percent are adenocarcinomas.

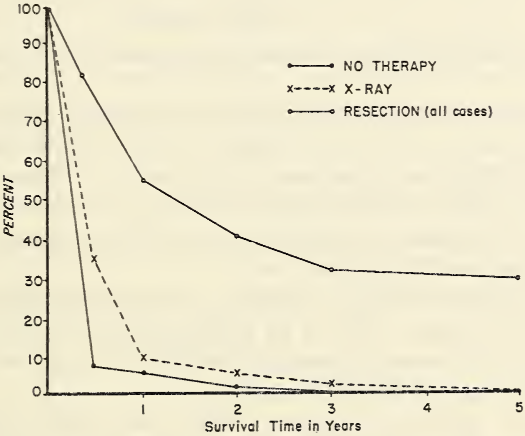
The therapeutic effects of radiation are valuable for symptomatic benefit, such as relief of bone pain, but I believe that X-ray therapy does not prolong the life of the patient with bronchogenic carcinoma. In fact, a series of cases reviewed some years ago indicated that patients treated with high-dosage therapeutic radiation were sicker, lost more weight, and died more quickly than those who were not treated.

Dr. Curreri: From the reports of Dr. Chalmers, Dr. Wolf, and Dr. Hyde, we have learned one important fact: that good supportive therapy is not any worse at the present time than chemotherapy plus X ray in far-advanced carcinoma, so that it is not morally improper to utilize control studies in lung carcinoma. There are other lesions in which certain agents are effective and, in these, chemotherapy is indicated.

It was postulated that if the lesion were totally or a major portion extirpated, the use of nitrogen mustard would destroy any remaining cells, either *in situ* or embolic, that might enter the venous stream or the pleural cavity. Dr. Hughes will give you the results obtained by the Veterans Administration group.

Dr. Hughes: Recently, we reviewed the results obtained in the treatment or the observation of 547 consecutive proved lung cancer patients admitted to the Kennedy Veterans Administration Hospital between 1947 and 1957, from the time the Veterans Administration established a thoracic surgery section there until the start of the surgical adjuvant study. There were 120 patients transferred from other hospitals; 427 were originally admitted to Kennedy; 209 are available for 5- to 12-year follow-up study. The survival for 5 years was 24 of the 209, which is 11.5 percent of the patients. Twenty-two of the 24 were the results of surgical treatment. There were 2 patients that did not have surgical treatment who survived 5 years. There were 59 that were resected more than 5 years ago, so that the 5-year survival was 37 percent of those resected. Since we were able to "cure" only 11.5 percent, there is a great need for improved treatment for the other 88.5 percent of patients.

Text-figure 5 shows the results of surgical and nonsurgical treatment a few years ago. The natural survival curve, no therapy group, shows at 6 months approximately 90 percent that did not survive, 10 percent of patients surviving at 6 months, a little less at a year, and very few surviving at 2 years. At that time we had approximately 32 percent 5-year survival after surgery. These cases were selected as carefully as possible because the results of surgical treatment probably depend mainly upon the selection of patients. The patient who has ability to control the cancer is the one who will benefit the most from surgery, and in the selection clinical observation probably gives the best chance to determine whether that patient still has the cancer under control. Surgery was done for cancer that could be completely removed, for patients with a life expectancy of a year, provided there was no distant metastasis.



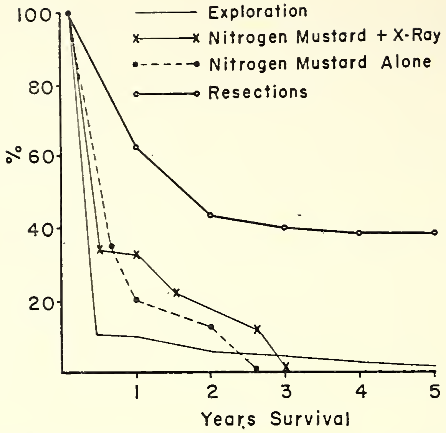
TEXT-FIGURE 5.—Results of surgical and nonsurgical treatment a few years ago.

Unfortunately, cases are not labeled cancer or noncancer, and there is often the problem of treating the patient with an undiagnosed pulmonary lesion.

The risk of surgery should be accurately evaluated. It is of little value to remove a cancer completely if a patient's heart will not tolerate the burden of the surgery, or if his remaining lung tissue will not properly oxygenate his blood. Most clinicians should preferably develop an aggressive attitude regarding undiagnosed lesions. When in doubt resect, because a cancer may be restrained for a time, but we can not tell for how long.

Text-figure 6 shows the results with 209 cases seen more than 5 years ago. There is some benefit from aggressive treatment. The 5-year survival from selected surgical cases is 37 percent and lobectomy has given results as good as pneumonectomy. If the tumor is completely removed, removing a lobe is as good as removing a lung.

From the use of X ray and nitrogen mustard there was 18 percent 2-year survival, and 1 patient survived 5 years. Of the untreated patients, 90 percent did not survive for approximately 6 months.

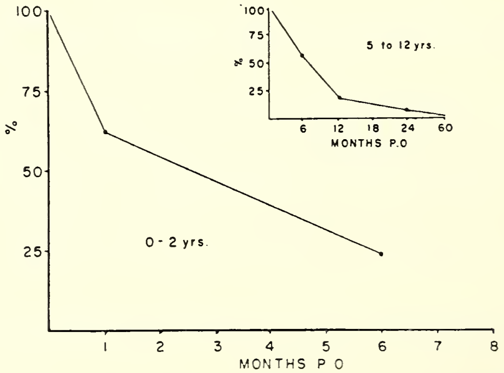


Text-figure 6.—Results of ancillary treatment in 209 patients.

The result after curative and palliative resection differ, the curative resection being one in which the surgeon considered the tumor completely removed with additional treatment unnecessary. There was 58 percent 5-year survival after curative pneumonectomy; 75 percent survival after lobectomy; after palliative resection plus nitrogen mustard or irradiation, there was 15 per cent 5-year survival.

One of 43 patients having exploratory thoracotomy followed by irradiation survived 5 years. One of 68 who received no treatment survived 5 years. If 1 to 4 percent naturally survived 5 years we tried to have them included with our resection cases.

Text-figure 7 shows one aspect of the cooperative adjuvant study. This percentage refers to the time of death related to total deaths.

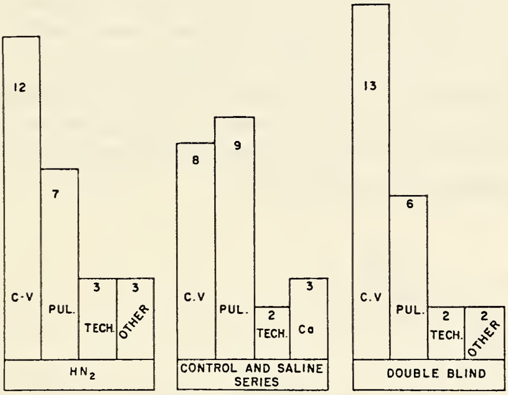


Text-figure 7.—Lung study. Data received to October 12, 1959: 182 deaths in series 0 to 5 years; 479 cases in study; 1,113 screened, including 189 double-blind.

Admitted to the study were 479 (43%) patients of 1,113 (100%) screened. Approximately 43 percent of all patients has been resected, which indicates definitely that we have a very aggressive attack. Surgery and adjuvant chemotherapy have been given to a large percent. In our Kennedy series, observed 5 to 12 years, we resected 27 percent of all patients. In the adjuvant study 16 percent did not survive (182 of the 479 had resection). Available for future study are 27 percent of all the patients (297 of 479 resected), which I think indicates that we are really aggressively applying adjuvant chemotherapy, and that in time we will have an answer as to whether nitrogen mustard is any good as an adjuvant to surgery.

In the adjuvant study, 37 percent of the total number of deaths occurred in the 1st month, 50 percent within 2 months, and 75 percent within 6 months. Text-figure 7 shows the Kennedy study with 5-to-12 year follow up; 93 percent of the deaths occurred in the first 2 years after operation; only 7 percent occurred during the next 3 years.

Text-figure 8 shows the cause of death of the postoperative mortality. The etiology is mainly cardiovascular and pulmonary in all 3 groups of the study—the double-blind, the control, and the series when 4 doses of nitrogen mustard were given.



TEXT-FIGURE 8.—Lung study: 30-day postoperative mortality; 479 cases, 0 to 2 years.

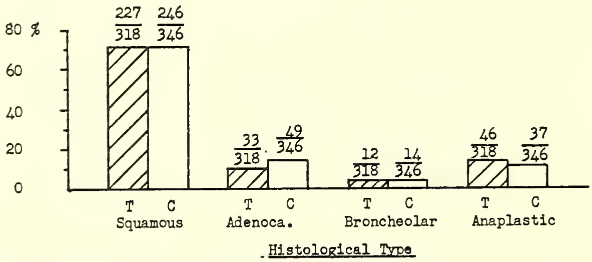
I do not know whether this is significant or not, but the cardiovascular lesions doubled the pulmonary causes of death in both series when nitrogen mustard was given. In the control and saline series, death from cardiovascular and pulmonary causes was about the same. Whether this is significant we do not know.

In summary, from our standpoint, surgery offers the most hope of cure for lung cancer for patients with resistance factors that localize the cancer, but it requires, on the part of the surgeon, mature clinical judgment and skillful execution. We feel that X ray and chemotherapy are valuable adjuncts, but that our methods of diagnosis and treatment must greatly improve if about 88 percent of these victims are to be greatly benefited.

Dr. Curreri: Dr. Hughes' statistics must be critically evaluated, for it should be realized that the surgical patients were, particularly in his first group, carefully selected and some of them appeared to have very limited disease. If these had been included in the nonsurgical group, probably the over-all results for the nonresected group might have been better. This may sound heretic from a thoracic surgeon, but we must be honest.

One must also consider in therapy the degree and extent of morbidity resulting from complications. In the original study we thought there were too many patients dying from pulmonary insufficiency and cardiovascular accidents. We seriously considered undertaking some basic studies in this regard. But as our study in the University group continued, it became apparent that the number of deaths from these complications was few when we considered that we were dealing with patients of advanced age with considerable emphysema, who required major surgery for the eradication of their disease.

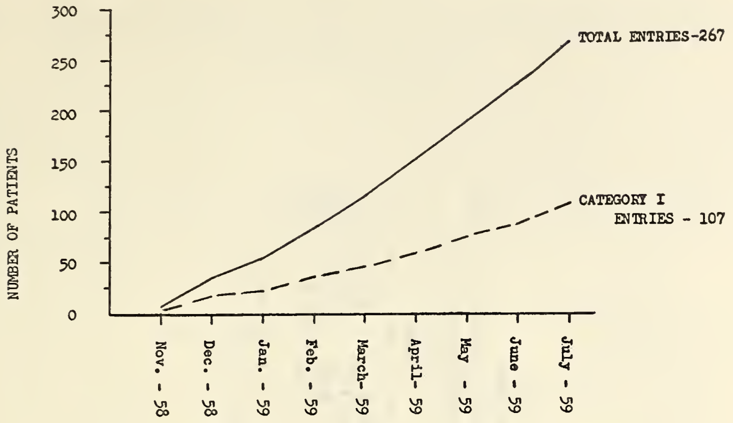
Briefly, the results obtained by the University group are shown in the following text-figures. The prognostication, made years ago, of an increase of squamous-cell carcinoma due to tobacco smoking has been fulfilled. Dr. Ochsner and Dr. De Bakey, approximately 14 years ago, reported an equal number of squamous-cell carcinomas and anaplastic type carcinoma. The slides of the University group were reviewed by at least 2 pathologists, with agreement in the vast majority. Seventy percent of our cases were squamous-cell carcinoma and the remainder were divided in the other three categories of adenocarcinoma, anaplastic, and alveolar carcinoma (text-fig. 9).



Text-figure 9.—Adjuvant chemotherapy lung study. Relative distribution of histological type of tumor classified by treatment group.

Text-figure 10 reveals that the mortality rate cannot be determined from 100 or 150 cases. In the course of our studies, some were disturbed that the mortality rate in 150 cases was about 25 percent, whereas the literature contains series in which mortalities of 5 percent for carcinoma of the lung and resections are reported.

Obviously most men that submit papers have had a run of good results; in like fashion, when they do not have good results they do not report it. But ours is an over-all picture. University groups often receive nothing



TEXT-FIGURE 10.—Percentage of each group of cases.

but the worst type of cases, since the readily resectable cases are handled by university-trained residents in their local areas.

Table 5 shows a cross-section representing 466 patients in the predouble-blind group. These cases were randomized with every other case treated either with nitrogen mustard or no compound. In this large group the true mortality rate is approximately 14 or 13 percent. There were 14 percent in the treated group and 13 percent in the control group. Actually there was the same number of deaths in both groups, but there were a few more control cases. This finding supports the need of adequate numbers for final evaluation. It was anticipated that the mortality rate for lobectomies would be 50 percent lower than the mortality for pneumonectomies.

Tables 6 and 7 show the results in the partially completed double-blind study. This group received a total of 0.3 mg. per kg. per body weight of nitrogen mustard as compared to 0.4 mg. per kg. in the original randomized groups. The high mortality of 21 percent in the treated and 15 percent in the control may be surprising and alarming. However, this represents the results in about 150 patients, and similar results were obtained in the original randomization study. The complications occurring in the randomization and double-blind studies were practically the same with but one sharp difference. The randomization group had 9 bronchopleural fistulae while the double-blind had 1. The latter is in keeping with the control groups.

Text-figure 11 demonstrates that nitrogen mustard as an adjuvant to surgery will not improve the survival rate. Patients in category I and II had the same survival rate at the end of 540 days whether they were in the treated or the control group. In category III, although statistically not significant, the statistics would indicate that nitrogen mustard as an adjuvant to surgery was deleterious to the patient.

If we interpose at 360 days the larger group of patients available for evaluation, we will rate no better results than at 540 days (text-fig. 12).

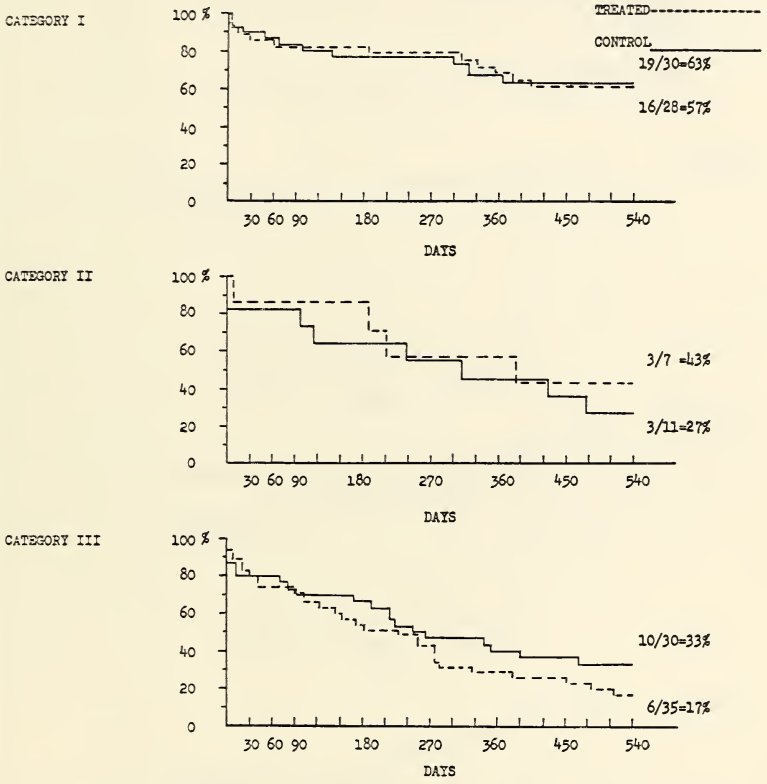
Table 5.—Adjunct chemotherapy lung study—predouble-blind phase

Category	Early postoperative mortality by surgical procedure and treatment group					
	Pneumonectomy		Lobectomy		Total	
	Treated	Control	Treated	Control	Treated	Control
I	4/52 = 0.08	8/53 = 0.15	2/32 = 0.06	1/46 = 0.02	6/84 = 0.07	9/99 = 0.09
II	9/27 = 0.33	4/37 = 0.11	0/8 = 0.00	1/13 = 0.07	9/35 = 0.26	5/50 = 0.10
III	13/69 = 0.19	12/74 = 0.16	3/32 = 0.09	5/23 = 0.22	16/101 = 0.16	17/97 = 0.18
Total	26/148 = 0.18	24/164 = 0.15	5/72 = 0.07	7/82 = 0.09	31/220 = 0.14	31/246 = 0.13

TABLE 6.—Adjuvant chemotherapy lung study—double-blind phase—August 31, 1959: Early postoperative mortality rates* classified by treatment group and final category

Treatment group	Final category			
	I	II	III	Over-all
Treated	7/34 = 0.21	4/19 = 0.21	7/32 = 0.22	18/85 = 0.21
Control	6/41 = 0.15	3/17 = 0.18	6/44 = 0.14	15/102 = 0.15

*Rate No. 6 as defined in Appendix I.



TEXT-FIGURE 11.—Adjuvant chemotherapy lung study—predouble-blind phase. Survival rates for patients operated from July, 1957, through December, 1957.

In short, one can say that this study has provided an excellent baseline for future studies wherein one can compare control and nitrogen mustard to any new agent.

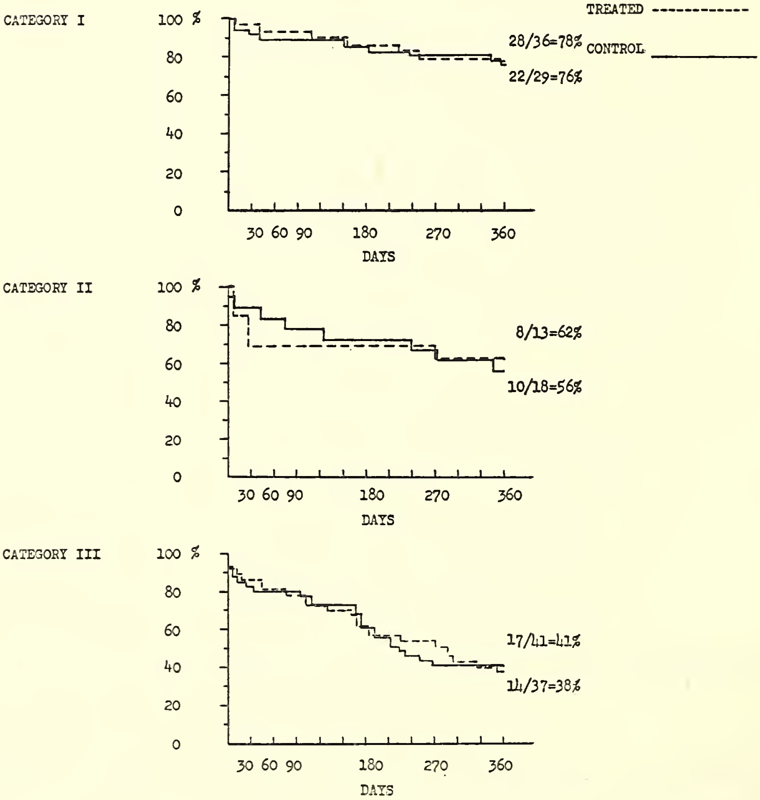
Dr. Hyde made the statement that if all patients with carcinoma of the lung were not treated, approximately 4 or 5 percent would survive 5 years. This would be in contradistinction to Dr. Hughes' findings.

In summary, lung carcinoma is still resistant to surgeon, radiologist, and chemotherapist. What does the future hold in terms of chemotherapy? As new agents are introduced, such as Cytoxan, consideration

Table 7.—Adjuvant chemotherapy lung study—double-blind phase

Causes of early postoperative deaths, classified by treatment group

Cause	Treatment	Control
Bronchopleural fistula	1	1
Myocardial infarction	1	2
Pulmonary coronary	2	1
Pulmonary embolism	1	2
Acute myocardial infarction	1	2
Cerebral vascular accident	1	1
Unknown	3	3
Others	9	4
Total number of deaths	19	16



Text-figure 12.—Adjuvant chemotherapy lung study—predouble-blind phase. Survival rates for patients operated from January, 1958, through June, 1958.

should be given to undertake pilot studies which may suggest a degree of effectiveness as an adjuvant to surgery in the treatment of lung carcinoma. Moreover, consideration must be given to combine therapy for possible potentiation through the use of two compounds or a combination of chemotherapy with X ray. The group at Stanford has been interested in combining 5-fluorouracil with radiation therapy and are presently excited about it.

Like Dr. Lee, I believe that while the picture does not look extremely bright now, it will change some day and then chemotherapy will make rapid advances.

Present Status of Investigation in Surgical Adjuvant Chemotherapy[1]

Chairman: DR. GEORGE E. MOORE, *Roswell Park Memorial Institute, Buffalo, New York*

Members of Panel:

Dr. Richard Dwight, *Veterans Administration Hospital, Boston, Massachusetts*
Dr. John P. North, *Veterans Administration Hospital, Dallas, Texas*
Dr. Rudolph G. Mrazek, *University of Illinois, Chicago, Illinois*
Dr. Robert R. Smith, *National Cancer Institute, Bethesda, Maryland*

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[1] Presented at Conference on Experimental Clinical Cancer Chemotherapy, Washington, D.C., November 11, 1959.

Dr. Moore: The adjuvant chemotherapy program seems quite logical to surgeons. I think no one is more aware of the limitations of surgery and X ray than surgeons, since they are painfully aware that cancer is not a local but a systemic disease in most instances.

Instead of being the result of animal study, as has been suggested, the adjuvant therapy studies were a logical development of conversations among surgeons who were concerned with a mutual problem. Although these surgeons were able to excise locally many neoplasms, they subsequently watched the patient succumb from distant metastases. The first clinical adjuvant chemotherapy studies were reported, more or less simultaneously, 7 years ago from Japan, Poland, Germany, and Russia.

The advantages of studying chemotherapeutic agents in conjunction with surgery are:

1. The host is still reasonably intact in those instances in which surgery is possible and, therefore, may react against malignant cells in addition to the surgical and chemotherapeutic help. The internists have stated very clearly that there is little doubt that terminal patients are poor subjects to use for the study of chemotherapeutic agents, since they would have a difficult time responding to therapy in any event. In addition, a minimal amount of tumor in the patient places comparatively minimal demands upon host-resistance factors. It is true, however, that surgical stress probably lessens host-resistance factors during the immediate postoperative period. Another criticism of the adjuvant studies has been that the added stress of toxic chemotherapeutic agents might worsen the balance between host and malignant cells. This may be particularly true in those instances in which a toxic agent is ineffective against cancer cells.

2. The adjuvant studies provide more honest studies. There is no question that individual assessments of chemotherapy are rather egocentric and biased by the enthusiasm of the investigator for a drug, especially "his drug." These individual ventures may be soul-satisfying to the investigator but, unfortunately, they do not provide the kind of data needed. Dispassionate figures on toxic complications and drug effectiveness, obtained according to a planned-objective protocol and based upon studies of a reasonable number of patients, are needed quickly to prevent clinicians from using the compounds without adequate information.

3. The adjuvant studies should yield an answer in the shortest possible time as to whether an agent is active or not. This is important since hundreds of patients will be given a particular compound shortly after a single prominent person writes a paper or gives a speech, with or without data, indicating that some kind of swamp water or other is good. Indeed, with a little magazine publicity, patients will demand such therapy. We hope to avoid premature ill-advised, uncontrolled use of these toxic agents by doing these studies. Approximately 5,000 patients have been registered in the several studies and 1,637 were eligible for the definitive trials.

I will briefly review the University studies. In the breast study, whose chairman is Dr. R. Noer, of the University of Louisville, there have been 346 patients. There were 8 deaths in the control group and 4 in the experimental. Forty-five complications have been listed in the treatment group and 36 in the control group. The only evidence of a beneficial result in this early phase of the study is a slight difference in local recurrences. In the control group there are 15 local recurrences out of 167 patients and 8 in a similar number of patients in the treatment group. Obviously, these data are not yet valid, but at least they are on the right side of the ledger.

The chairman of the colon-rectal group is Dr. W. Holden, of the Western Reserve School of Medicine. This group has 391 patients. There has been 11 percent deaths in the control group and 11 percent in the treatment group. Postoperative complications number 55 in the control patients and 42 in the treatment group. The deaths subsequent to a 30-day period after surgery would be most apt to reflect a good or bad effect of the chemotherapy. There have been 20 deaths in the control series and 12 in the treatment group. So the survival trend is favorable, and is still statistically significant.

Dr. Anthony Curreri, of the University of Wisconsin, is chairman of the lung adjuvant study. There have been 13 deaths in the control group, and 14 in the treated group. There is no difference in the survival curves up to the present time.

Dr. W. Longmire, of the University of California at Los Angeles, is the chairman of the gastric adjuvant group. The mortality rate in the control group has been 18 and 22 percent in the treatment group. This is relatively high in contrast to the 4 to 8 percent mortality rates reported in the literature. Complications occurred in 58 patients of the control group and in about 100 patients in the treatment group. Thus, even though there is very little difference in the mortality rate, there have been definitely more complications in the treatment group. I think this accurately reflects the adverse effects of chemotherapy. For example, there are 9 instances of pneumonias listed in the treatment group, 5 in control patients; 16 wound infections in the treatment group, and only 9 in control patients. These are preliminary figures and include patients treated with the higher dosage.

The survival curves of the gastric study are interesting. There have been 54 deaths after the 30-day postoperative period in the control group and 46 in the treatment group. The survival-rate curves favor the treatment group. The curves for both the treatment and the control group are identical for about 12 months and then start to separate. This coincides almost exactly with the sort of curve that we would predict for the following reasons: (1) These compounds are not very good compounds. They are mediocre as miracle drugs and cannot be expected to destroy large amounts of residual cancer. Therefore, we would not expect to see any drug effect until all patients with gross residual cancer following operation died. (2) Only those patients that had only residual scattered cancer cells or clumps of cells in the blood, lymphatics, or body cavities might be affected by these compounds. Thus, after the unfavorable prospects for survival die, there should be a separation of the survival rates of the patients, with a more favorable chance in the control and treatment groups. This is the sort of result that we hope we are getting in two of the studies.

The best study of all should be that involving carcinoma of the ovary, since a majority of advanced ovarian tumors definitely respond to several of the alkylating compounds presently available. Unfortunately, we have encountered another serious complication in this study. Once the gynecologists found from Dr. Masterson's preliminary study that the alkylating agents worked quite well on ovarian carcinoma, some refused to participate in the definitive studies because, they said, it was not fair to the patient. This has reduced the number of groups available for this study.

Now that we have briefly discussed the status of the clinical studies material, some of the exploratory experimental and clinical studies which will affect future studies done by surgeons will be presented.

* * *

I can substantiate these findings of the group at the University of Illinois, as we have done parallel studies. We have looked for tumor cells in the blood of about 3,000 patients and have found that a large percentage of patients with advanced cancer have tumor cells in the blood. There is little doubt that the cells are also released into the blood from some kinds of early, operable lesions. We do not know whether there is any rhythm in the exfoliation of cells into the blood and lymphatic vessels or not. Certainly normal movements of the organs and external trauma would probably be expected to increase the chance of dislodging tumor cells which have invaded into venules. Approximately 80 percent of the blood samples removed at the time of operation for lung cancer have tumor cells. Similarly about 50 percent of blood specimens obtained at surgery from carcinoma of the stomach and about 30 percent of the specimens associated with colon carcinoma contain carcinous cells. Dr. Watne, of our group, has cannulated the thoracic duct in an attempt to see whether the tumor cells are getting into the circulation directly by invasion of the blood vessels or by way of lymphatics.

It is interesting that whereas lymph specimens from patients with carcinoma of the lung have large numbers of tumor cells, similar specimens from patients with even advanced carcinoma of the colon rarely have tumor cells.

The effect of stress on the spread of tumor cells in experimental animals is noteworthy. Nevertheless, we should remember that most of these studies, ours as well as others, use implanted tumors, and regardless of how close immunologically the implanted tumor is to the host there still is a chance of host-tumor resistance, which may differ from that accompanying spontaneous tumors.

Nevertheless, there are two articles in the literature which indicate that the addition of cortisone to animals with spontaneous tumors results in more metastases. Recently Pelner has reported that patients who have been given large amounts of cortisone for nonmalignant disease have developed an exacerbation of their malignant disease. Dr. Smith, of the National Cancer Institute, has worked on many aspects of these problems and will relate the experimental studies carried out at the National Cancer Institute.

I would like to summarize a series of clinical studies concerned with the modes of cancer spread being pursued at Roswell Park. Previous mention has been made of the spread of tumor cells in the regional and peripheral blood stream, and the lymphatics. Our impression from these studies is that there is a characteristic pattern of cell spread into the blood or lymph from different kinds of tumors. In general these seem similar to the pattern of metastases which have been described for them. In contrast to findings of Engell, we have preliminary data showing that there is a longer survivorship of patients in whom tumor cells have not been demonstrated in the blood than those with comparable lesions in whom circulating tumor cells were demonstrated.

Cancer cells also exfoliate into the body cavities. At the time of the operation, we have performed washings of the thoracic and of the peritoneal cavities, and then examined smear preparations for tumor cells. As an example, tumor cells were found in 30 percent of such washings from the patients with carcinoma of the colon and 50 percent of those deemed inoperable. The findings in patients with carcinoma of the ovary are even worse. Approximately 66 percent of those with tumors confined to the ovary had positive washings. The frequency with which tumor cells were found rose to over 80 percent in patients with extension of disease outside of the ovary. Again, these results are similar to the experience of gynecologists that approximately 50 percent of all patients with carcinoma of the ovary limited to the ovaries die within 2 years. This is ample illustration of a sort of tumor that should be attacked by both local and systemic application of chemotherapeutic agents.

Further mention of the possible adverse effects of chemotherapy must be made. Both experimental and clinical experience indicate that if a toxic compound which is ineffective, or only slightly effective, against a carcinoma is used, the bad effects on the patient may outweigh the beneficial effects gained from destroying only a small portion of the tumor. At the present time we have inadequate data upon which to make proper selection of drugs and dosage schedules for different kinds of tumors. Thus, in all surgical studies involving potentially curable patients by orthodox techniques, conservatism in the use of adjuvant chemotherapy is mandatory.

Future studies contemplated by the surgeons include more studies of pathogenesis, studies of hormone inhibitors, such as the use of thyroxin in thyroid resection, endocrine gland ablation, and X-ray and surgery combination therapy. It is hoped that some of the latter studies will settle controversies which have raged for decades. These and similar studies will afford us the continuing opportunity to train surgeons in modern techniques of therapeutic evaluation, improve the care of cancer patients, and in general prepare for the testing and use of better anticancer drugs.

Question: In the ovary adjuvant program, were only patients with primary, resectable, ovarian lesions considered?

Dr. Moore: That is correct. Only patients in whom we think we have removed all gross cancer are entered in the definitive adjuvant studies.

Question: Were the survival figures of the treatment and control groups in the gastric study significantly different or were you indulging in wishful thinking?

Dr. Moore: Surgeons are always a little euphoric; it is hardly worth mentioning. No, the curves are not statistically significant although they favor the treatment groups at this time.

Question: In view of the toxicity of full doses of alkylating agents and of the lack of effect on survival, would you advise the practicing surgeon to avoid the use of adjuvant chemotherapy?

Dr. Moore: We definitely would. Gentlemen of the panel, are we in agreement?

Dr. Dwight: I think we are certainly in agreement on that. So far the adjuvant study has shown that one thing, and perhaps nothing else. If it had not been a cooperative study with many hospitals involved, perhaps we would not have realized that the dosage we were using was very dangerous. The virtue of the cooperative study was that we found out before we had gone too far that it was a dangerous thing and no one should do it alone; it would take too long to discover that you were doing harm.

Dr. Moore: Unfortunately, we have found in going across the country that many surgeons think that now in order to be "up-to-date" they must give chemotherapy to all cancer patients. One surgeon related that in conjunction with a pelvic exenteration procedure he not only had given nitrogen mustard systemically but put Chlorpactin in the wound. This chemical solution is being used widely, probably because it is the only compound being advertised widely. We would mention these things only to deplore them. We do not think these chemotherapeutic compounds should be used by the practicing surgeon until we have some evidence that their beneficial effects outweigh their adverse effects.

Dr. Dwight: The early mortality in the Veterans Administration study which led to a change in dosage later is something that is going to be discussed by Dr. North, who is the chairman of the Veterans Administration study. The thing that I was prepared to talk about, and which is in a way a corollary of that, is the hematology in the early days of the Veterans study.

You have heard us refer to the "full-dosage series" in both the University and the Veterans adjuvant studies. We started using what we called a "full dose" of chemotherapy; 0.4 mg. of nitrogen mustard per kg. in lung cases, 0.8 mg. of thio-TEPA per kg. in the stomach and colon cases as an adjuvant to surgery. It was found in the Veterans Administration study that this produced a rather high mortality and we later started a double-blind study using three fourths of the dose.

When we were using the full dose, we unfortunately did not do as many hematologic studies as might have been desirable. In our protocol we required white counts on the 7th and 14th postoperative days. We found that on the 7th postoperative day in treated, nonfatal cases the median white count was 8,000 compared to 14,000 in the controls. And on the 14th day, it was 6,000 compared to 11,000 in the controls. By the 21st day the median white counts in both groups were approximately the same.

It was also observed that 11 percent of the treated cases on the 7th day and 36 percent on the 14th day had white counts below 5,000, whereas none of the controls did. On the 7th day, 40 percent of the controls had white counts over 15,000 compared to 2.2 percent of the treated cases and on the 14th day, 28 percent of the controls had white counts over 15,000 compared to none over 15,000 in the treated cases. The hemoglobin and the platelet counts showed no difference between the two. This suggested that, while there were very few absolute leukopenias, the treated cases were unable to develop a high white count immediately after surgery.

Since beginning the double-blind series, in which the patients are getting three fourths of the dose we previously used, we have been doing white counts on the 1st, 3d, and 5th postoperative days as well as the 7th and 14th; and on the 21st and 28th days when the patients remain in the hospital as long as that. There has been no significant difference between the treated and nontreated cases, and this is true in the 1st week and also on the 25th to the 29th day. White counts under 4,000 have not been observed on the 1st and 2d postoperative days, only 1 has been observed on the 7th to the 10th day, and 6 on the 25th to the 29th days in 300 cases in our present series. This all suggests that whereas the full dose of nitrogen mustard and thio-TEPA not infrequently produces at least a relative leukopenia, three fourths of the dose rarely does so.

As for the complications in the full-dosage series, in 37 treated cases in the stomach series there was a 57 percent incidence of complications compared to 46 percent in the controls. This is not a very striking difference, but when the palliative cases were considered, and perhaps again I should explain that in all sites we randomized palliative cases and cases which we thought we were curing in different series, there were twice as many complications in the treated ones as in the controls. And in the colon series there were nearly 3 times as many complications in the palliative cases.

Of the complications, the most striking were the cardiovascular, which were 5 times as common in the treated stomach cases and 4 times as common in the treated colon cases.

In the full-dosage series we have seen no difference in the long-term survival, "long term" being in this instance a year, between the treated cases and the controls. The only slight ray of hope is that, in the palliative stomach cases, a small group, the treated cases have made up for the higher postoperative mortality, and the survival curve which was running below that of the control cases is now slightly above it. At most, this is just a straw in the wind.

Dr. North: In the discussion of these surgical adjuvant studies, a point should be made, I believe, that these apply to cases which have excisional surgery of the tumor, to the best of the surgeon's ability. They do not include any cases in which the primary tumor was not removed and in which simply palliative operations were done. When we refer to palliative surgical adjuvant cases, we mean the cases in which the surgeon felt quite certain that he was leaving tumor behind in the course of the resection.

Now, a good deal has been said here and elsewhere about the perhaps unique experience we encountered in the early months of this study. When the study had been going on about 6 months, we began analyzing our results and found that the mortality during the first 30 days after operation was considerably higher in the cases that received adjuvant nitrogen mustard (in the lung series) or thio-TEPA (in the colon or stomach resections). These discrepancies were not merely trivial ones, but seemed to us rather disturbing. They were of the order in the lung series of a 7 percent operative mortality in the control cases and 22 percent in the treated cases. In the stomach study, there was a 10 percent mortality in the first 30 days for the controls and 23 percent in those that received thio-TEPA.

We were further disturbed by the fact that, although these control mortality figures were quite consistent with the mortality which had been present in the same 21 hospitals previous to the study, the treated mortality figures were much higher. We had no choice at that point, we felt, except to stop this study and revert, for the time being, to a continuation of the study with all patients receiving a placebo or normal saline. Under that regime, the mortality reverted to the level of the previous control mortality and the level obtained in the same hospitals prior to the study.

During this interim period, we analyzed the group thoroughly in every way that we could imagine to see if there had been any bias entering into the randomization procedure, to see if by chance any great number of elderly people, of if an excess of poor-risk patients were in the treated group. We could find no such differences. The causes of death and the manner in which patients died were approximately the same in both groups. You could pair them off and find one in one group and one in the other. We were forced to conclude that the difference in the mortality figures had something to do with the dosage administration of the drug. This has been further confirmed by the fact mentioned by earlier speakers that when we reverted to a ¾ dose, 0.3 mg. per kg. of nitrogen mustard and 0.6 mg. per kg. for thio-TEPA, the mortality became equal in both controls and treated patients. Our only explanation for this, and it may or may not be valid, is the long-recognized fact that if X ray is given concomitantly with surgery, or in close proximity to surgery, the patients do not tolerate as high a dosage of radiation. The same may be true of chemotherapeutic agents. If at the time the patient has a major surgical resection, he is given what would ordinarily be considered a conventional dose of an alkylating agent, he may not be able to tolerate this combination. Our experience is not in accordance with all similar studies but it did lead us to change our dosage level at an earlier time than we would ordinarily have done, even though we had not collected a sufficient number of cases in the original group for full evaluation.

Dr. Mrazek: At the University of Illinois we have been interested in the clinical adjuvant chemotherapy studies, in which we are studying cancer of the breast, colon, and rectum. We are using nitrogen mustard in a dose of 0.4 mg. per kg. of body weight (including the initial dose given at surgery). During the 1st postoperative year, we give 3 additional courses of nitrogen mustard (at 4-month intervals) and during the 2d postoperative year we give thio-TEPA at monthly intervals, if there is no leukopenia.

We are interested also, clinically, in wound irrigation with agents that will destroy neoplastic cells which may be seeded into the wound during an operation. We are also studying cancer cells in the peripheral blood. Recently, we have noted that stress of various types including an operation will decrease the resistance of a rat to Walker 256 carcinosarcoma cells.

We are also conducting tissue-culture studies, trying to assay various anticancer agents against *in vitro* human and animal tumors.

Adjuvant use of nitrogen mustard.—We currently have 100 patients in our mastectomy series, half of whom have received nitrogen mustard, half of whom serve as controls (table 1). This series began 3½ years ago. The most recent studies are just a few weeks old, however. To date, we have noted 10 recurrences in our patients treated with nitrogen mustard, compared to 15 in our controls. These have occurred in bone, nodes, the other breast, liver, or lung, but we have not seen local recurrences in the patients treated with nitrogen mustard. The average time of occurrence of these recurrences was 13 months in the treated patients and 14 in their controls.

We have had 2 deaths due to carcinoma in the treated patients as opposed to 8 in their controls. Again there was no marked difference in the time of death.

TABLE 1.—Cancer of the breast: mastectomy and adjuvant chemotherapy with nitrogen mustard

100 Patients—50 treated, 50 controls

	Nitrogen mustard	Control
Recurrences	10	15
Location:		
Bone	3	4
Nodes	3	7
Other bresst	2	1
Liver or lungs	2	1
Local	0	8
Deaths due to cancer	2	8

In the colon-rectal series, we now have 70 patients; half of these have received nitrogen mustard. In this group there is no great difference in recurrences. We have 9 recurrences in patients treated with nitrogen mustard and 7 in their controls. There have been 5 deaths due to carcinoma in the treated patients and 3 in their controls. There was no marked time difference between the two groups.

Again I would like to emphasize that we are giving nitrogen mustard in repeated doses during the 1st year and thio-TEPA during the 2d postoperative year, if the bone marrow will permit it. We give nothing after this period.

Irrigation of wounds with anticancer agents to kill cancer cells.—We are currently interested in wound irrigation with cancerocidal drugs to reduce the possibility of implantation and growth of cancer cells. Dr. McDonald in our laboratory is studying various compounds for this use (table 2). This was done by implanting Walker 256 cells in a pouch on a rat's abdomen. After 1 hour the pouch was widely opened and irrigated with various solutions. In the controls there was a 95 percent take. Saline, distilled water, oxytetracycline, and heparin increased the take slightly. Other compounds were effective in increasing sequence.

We found nitrogen mustard in a concentration of 1 or 2 mg. percent, Chlorpactin 2 percent, or sodium hypochlorite 0.5 percent, buffered to *p*H 9, to be most effective. Clinically, we are currently using the buffered sodium hypochlorite solution. We hesitate to use nitrogen mustard in patients already receiving the compound as adjuvant therapy because of the danger of added toxicity. We found that Chlorpactin in a concentration of 2 percent inhibited wound healing and was too toxic for clinical use.

Cancer cells in the blood.—We have been extremely impressed by the demonstration of cancer cells in the peripheral blood and our group, Roberts *et al.*, have now studied over 600 patients for these cells. In the first 328, these cells were studied by an albumin flotation technique. Currently we are using a streptolysin technique. In the first 328 patients, we were able to demonstrate cells in 23 percent of the curable patients, whereas they were seen in 33 percent of patients with advanced incurable tumors. If the study were restricted to peripheral blood we found somewhat lower percentages, 20 percent in curable patients and 29 percent in those with incurable lesions and 37 percent of the incurable lesions (tables 3 and 4).

Table 2.—Experimental irrigation of wounds inoculated with Walker 256 carcinosarcoma cells 1 hour previously*

	Percent tumor
Control	95
Saline, isotonic	100
Oxytetracycline	100
Distilled water	100
Heparin, 10 mg. percent	100
Saline, 3 percent	90
Chlorpactin, 0.5 percent	86
Phenol, 0.1 percent	86
Sulfadiazene, 1 gm. percent	83
Chloroazodine, 0.05 mg. percent	80
Benzalkonium, 1:1000	71
Hg bichloride, 1:1000	66
Ethyl alcohol, 95 percent	57
Chlorpactin, 1 percent	46
Chlorpactin, 1.5 percent	36
Lugol's solution (½ strength)	23
Sodium hypochlorite, 0.5 percent	13
Formaldehyde, 0.3 percent	12
" 0.4 percent	10
Styryl-quinoline, 10 mg. percent	10
" " 15 mg. percent	9
Formaldehyde, 0.5 percent	8
Sodium hypochlorite, 0.5 percent, buffered to *p*H 9.0	8
Chlorpactin, 2 percent	6
Nitrogen mustard, 2 mg. percent	4
" " 1 mg. percent	4

*G. W. McDonald *et al.*: Wound irrigation in cancer surgery. Ann. Surg. In press.

Table 3.—1276 Blood samples, 328 patients with malignant disease, and 35 patients with benign disease* †

	Number of patients	Number of patients (*positive*)	Positive (*percent*)
"Curable"	105	24	23
"Incurable"	223	73	33

*Long *et al.*: A. M. A. Arch. Surg. In press.

†Two of these patients were thought to have malignant cells in the circulating blood, *i.e.*, false positives (0.55% of series).

We have been interested in the response of these cells to chemotherapeutic agents and in these patients we have tried thio-TEPA, Nitromin, and nitrogen mustard, to see what effect these drugs would have on the circulating cells. In a group of patients we found, with one exception, that cells either decreased or disappeared after the drug was given.

Several patterns of cell levels in the blood seem to appear. One was a level where no cells were found before surgery. There was a spike of cells during a manipulation of surgery and no cells could be found afterward.

TABLE 4.—Summary of 297 cancer patients—peripheral blood (antecubital vein) by organ system*

	"Curable" patients		"Incurable" patients	
	Number of patients	Positive (*percent*)	Number of patients	Positive (*percent*)
Digestive tract	20	0	65	23
Genitourinary tract	11	27	14	57
Breast	29	24	22	23
Female genital tract	7	14	28	32
Miscellaneous	23	30	78	28
	90	20	207	29

*Long *et al.*: A. M. A. Arch. Surg. In press.

Another pattern is that of cells before surgery, a spike during surgery, and no cells after the removal of the tumor. A third pattern seems to be that of cells before and during surgery, a decrease after surgery, but a return to preoperative levels.

Role of stress in decreasing the resistance of animals to cancer cells.—At a laboratory level we have been impressed with the role of stress in an animal's resistance to implanted tumor. We defined stress as surgical stress, anesthetic stress, simulated stress with steroids, cold stress, and the stress of liver damage. Four parallel experiments are shown in table 5. They reveal the effect of operative stress on the take of Walker 256 cells in the rat; in all these rats 25,000 aged cells were injected subcutaneously. All groups except one were anesthetized. Without exception, an increased incidence of "take" in animals exposed to the stress of celiotomy or of other surgery is seen.

TABLE 5.—Role of operative stress on the take of Walker 256 carcinosarcoma cells*

25,000 Aged cells injected subcutaneously; celiotomy, wound closure in 45 minutes

	Control (*percent*)	Takes operation + Nembutal anesthesia (*percent*)
Buinauskas *et al.*	31. 6	61. 1
Trevino, Gines *et al.*	15. 2†	66. 5
Slawikowski	35. 1	51. 3
Creath *et al.*	14. 8	40. 4

*W. H. Cole *et al.*: Dissemination of Cancer. New York, Appleton-Century-Crofts, Inc. In press.
†No anesthesia.

Another study was done by Trevino to evaluate the effect of anesthetic stress (table 6). Again 25,000 cells aged 12 hours to reduce the takes well below 100 percent were injected subcutaneously. The control animals were not anesthetized. When rats were anesthetized with Nembutal, the incidence of take was 35 percent as compared with 15 percent in their

TABLE 6.—Role of anesthesia on resistance of rats to Walker 256 carcinosarcoma cells*

25,000 Aged cells injected subcutaneously		
	Control rats No anesthesia (*percent*)	Anesthetized rats (*percent*)
Nembutal, 30 mg. per kg.	15. 2	35. 7
Ether, 1 hour	24. 4	41. 0

* Trevino *et al.*: Unpublished data.

controls. When they were anesthetized with ether for 1 hour, the take was 41 percent as compared with 24 percent in their controls.

The role of hypothermia and cold stress on the take of Walker cells is illustrated in a study by Dr. Griffith. The control animals were not exposed to cold. In one group, rats were anesthetized and their body temperature lowered to 75 degrees for 2 hours. The percentage take in these refrigerated animals was 92 percent as compared with 57 percent in their controls. In another group, the rats were not anesthetized. They were put in a cold room at 21° F. for 6 hours. In this case, the chilled animals sustained takes in 96 percent, compared to 72 percent in their controls. These unanesthetized animals were given food *ad libitum.* They succeeded in eating 16.5 percent of their body weight during the period they were being chilled.

The hyperthyroid state which may represent a stress also has been studied. Humphrey and dePeyster have rendered rats hyperthyroid with tri-iodothyronine. Takes occurred in 80 percent of their control animals. In the hyperthyroid animals, the take fell to 59 percent. We have also tried to evaluate the effect of liver damage on the resistance of rats to Walker 256 cells. These rats were given carbon tetrachloride subcutaneously to damage their livers. Cells then were injected intraportally. The percentage take in the animals with liver damage was 49 percent (table 7). The take in their controls was 18 percent.

TABLE 7.—Effect of liver damage on the takes of intraportal Walker 256 carcinosarcoma cells*†

	Number of rats	Liver takes (*percent*)
Liver damage‡	69	49
Controls	50	18

*Chan and McDonald: Unpublished data.

†Cells aged 12 hours to reduce percent of tumor takes.

‡CCl_4 subcutaneously for 3 days prior to tumor inoculation.

We have attempted to block this stress by the administration of a compound called amphenone, which decreases the formation of steroids in the adrenals. It was given in a dose of 100 mg. per kg. of body weight. However, these experiments revealed no reduction in the take of injected cells after the amphenone.

The final topic is that of tissue culture of cancer cells *in vitro*. Dr. McDonald is carrying this out in roller tubes, hoping that this will be an instrument whereby we can evaluate cancerocidal drugs *in vitro* and possibly select the particular drug for the particular patient.

Dr. Smith: The ability of the surgeon to control cancer is dependent upon two of the distinguishing characteristics of cancer: (1) its ability to invade normal adjacent tissue, and (2) its ability to produce secondary tumors at distant sites; *i.e.*, metastasis. To extend the scope of surgical resections may assist in the local control of cancer, but if distal dissemination has occurred, the patient is incurable. The surgeon, therefore, is very interested in the pathogenesis of metastasis and in any factor that may alter the implantability and growth of tumor emboli.

In the past, studies of metastatic cancer have largely centered on autopsy observations. This gave an understanding of the end results of metastatic process, but did not demonstrate the biologic factors involved. These anatomic studies, enhanced by the application of Verchow's principles of cellular pathology, led to an anatomic concept of the pathogenesis of disseminated cancer; *i.e.*, small tumor emboli are carried in lymph and blood vessels from the site of the primary tumor to distal organs where they implant and form secondary tumors, metastasis.

In more recent times, studies have centered around experimental methods, which make it possible to explore the underlying mechanisms or dynamics of metastasis. In studies of human cancer, this becomes difficult because of the long time interval involved, the inability to alter biologic factors which may influence metastasis, and the heterogeneity of cancer as it occurs in man. Clinical studies of wound washings and of local and peripheral blood for circulating tumor cells do assist in the understanding of the problem of metastasis, for it delineates the anatomic extent and site of disease as it is developing. Studies of wound washings have demonstrated the frequency of wound contamination by tumor cells after definitive cancer surgery (*1*). Twenty-six percent of operative wounds studied had tumor emboli demonstrated in wound washings. An additional 14 percent were reported to contain "suspicious" tumor emboli.

Finding these cells led the surgeon to search for means of preventing growth of these implants. A number of compounds were studied in artificial tumor-seeded animal wounds. Five-tenths percent formaldehyde prevented growth of tumor emboli in almost 100 percent of the artificially seeded animal wounds (*2*). It was used in a series of human cancer surgery (*3*). Preliminary study of a group of human cervix uteri cancers treated by pelvic *en bloc* surgical resection gave negative results. Survival experiences based upon life-table analysis and local recurrence experience were about the same as those observed in the control groups. Similar results were observed in a series of patients with cancer of the head and neck areas treated by radical surgical resection followed by a wound wash of formaldehyde.

Several factors have been observed which suggest that local wound chemotherapy alone will probably not influence the postoperative course of human cancer. Further studies of patients that have had wound washings taken after definitive surgery reveal that the finding of tumor emboli in such washings has little significance in determining prognoses. The incidence of local recurrent cancer in patients that had negative wound washings was about the same as that observed in the positive washing group (*4*). A similar observation was made by Engell (*5*) in his classic study of circulating tumor cells in colon cancer. Five years after surgery, 51 percent of his cases that had circulating tumor cells in the blood draining the tumor, at the time of surgery, were alive and free of cancer as compared with 49 percent of those that had no tumor cells in their local blood.

Another factor that leads one to doubt the value of chemotherapy limited to the local wound is the observation that cancer patients do not die of uncontrolled local disease alone, but more and more have disseminated cancer as well. Early studies of cervix cancer showed that patients at autopsy had cancer metastases of the pelvis in only 20 percent of the cases. In series of autopsied head and neck cancers, 10 to 20 percent had metastases below the clavicle. More recent autopsied patients did not conform to this experience. Of the patients with cervix cancers autopsied at the National Cancer Institute 80 percent had widespread metastases, and 50 percent of the patients with head and neck cancers had disseminated disease. It would seem that as means are developed to assist patients so that they do not die of their local cancer, the neoplasm has time to implant and grow at distant metastatic sites which lead to the death of the patient. Therefore, one is led to the problem of studying means to prevent the implantation and growth of tumor emboli that are known to be disseminated not only in the operative wound but throughout the entire body.

The use of adjunctive chemotherapy after definitive surgery for cancer of various sites has been discussed in the previous papers. Such studies are designed to demonstrate the effectiveness of a drug in preventing dissemination of cancer at the time of surgery. In order to understand better the problems involved, studies in experimental animal systems have been devised. This paper will review a study which suggests a controlling factor in the effectiveness of thio-TEPA on the development of artificial metastasis with S91 melanoma in mice. The experimental thio-TEPA study, reported by Kramer (*6*), demonstrated an accentuation of effectiveness of the drug when given 48 hours after the time of implantation of tumor emboli. In an effort to determine if any factor could be identified which could account for this time relationship in the effectiveness of thio-TEPA, Dr. David Kinsey, of Ohio State University, Columbus, performed the following study:

DBA mice were injected intravenously with a cytosieved mixture of S91 melanoma. Two animals from the group were killed at 6, 12, 24, 36, and 48 hours and at 3, 5, 10, and 21 days after inoculation. One lobe of the lung of each animal was serially sectioned and studied independently by two observers: Dr. David Kinsey and Dr. Edward Price, pathologist at the National Cancer Institute. They reviewed each section in an attempt to find evidence of change in position, or the character of the tumor emboli trapped in the lungs, that could account for the time relationship of the change in effectiveness of thio-TEPA observed by Dr. Kramer in his experiments. A summary of their observations is given:

Immediately after intravenous injection of tumor inoculum, the embolic tumor cells were located within the alveolar septal capillaries, usually as single cells and an occasional clump. Six hours after inoculation, the emboli were again seen within the septal capillaries, and some were observed within the lumen of small peripheral vessels. At 12 hours, growth was seen within small peripheral vessels and septal capillaries. From this point until 36 hours after inoculation, no significant change was observed. Prior to this time all emboli were found to be intravascular in position. From 36 to 48 hours, a gradual shift from intravascular to extravascular position was observed. Endothelialization of tumor emboli and growth of the tumor within the pulmonary parenchyma appeared at day 3. Intravascular collections of tumor cells were still observed on day 10, but by 21 days no intravascular emboli remained. A summary of these observations is given in table 8.

This study demonstrated that the 48-hour peak in effectiveness of thio-TEPA, observed by Kramer, correlates with changes of position of the tumor emboli from an intravascular to an extravascular position. This would suggest that the effectiveness of the drug could be related to the concentration bathing the implants, being greater when the emboli remain intravascular in position and becoming less effective as the concentration around each cell becomes less as the implants grow to an extravascular position.

In summary we can conclude that in an experimental transplantable tumor animal model, the effectiveness of a chemotherapeutic agent can be correlated with anatomic position of the tumor emboli. In this instance, the effectiveness of thio-TEPA in preventing the growth of lung metastases was significantly decreased when the implants grew away from an intravascular position. As the implants became larger, the drug became less effective. This type of information would support the use of surgical adjunctive chemotherapy, for after surgery circulating tumor cells should disappear and implants be of the smallest size.

TABLE 8.—Location of tumor emboli in mouse lungs at various times after intravenous inoculation of S91 melanoma

Location	Hours						Days			
	0	6	12	24	36	48	3	5	10	21
Intravascular capillaries	+++	++++	+++	++	++	++	+	+	+	0
Small peripheral vessels	0	+	++	++	+++	+++	+++	+	+	0
Large central vessels	0	0	0	+	++	++	+++	+++	++	0
Extravascular	0	0	0	0	+	+	++	+++	+++	++++

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Use of Alkylating Agents and the Future of These Agents[1]

Chairman: DR. RALPH JONES, Jr., *Jackson Memorial Hospital, Miami, Florida*

Members of Panel:

Dr. C. Gordon Zubrod, *National Cancer Institute, Bethesda, Maryland*
Dr. David A. Karnofsky, *Memorial Center, New York, New York*
Dr. Bernard R. Baker, *Stanford Research Institute, Menlo Park, California*

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[1] Presented at Conference on Experimental Clinical Cancer Chemotherapy, Washington, D.C., November 11, 1959.

Dr. Jones: We will deal with the present status and the future prospects of the alkylating agents. I have requested that we confine our discussion to the bis-β-chloroethyl and the ethyleneimine types of alkylating agents because we have the most information about these two types.

The present clinical status of the alkylating agents is well known. They are a group of chemotherapeutic agents that are generally useful for the management of patients with malignant lymphomas; they have well-demonstrated activity and general utility in the management of chronic leukemias; well-demonstrated and thoroughly documented therapeutic effects against carcinoma of the ovary; they have readily demonstrable therapeutic activity against carcinoma of the lung, but this is of limited usefulness to the patient; they have limited therapeutic activity in some patients with carcinoma of the breast; and, in rare instances, have been reported to produce striking temporary regression of other types of human cancer. As Dr. Baker pointed out, more than 600 of these agents have been made. The question has been raised repeatedly whether all alkylating agents are the same or whether they are different. This is one of the questions that we shall consider.

In conducting a search for better agents for the treatment of a disease, one must proceed (1) to search constantly and tirelessly for new agents representing different structures not previously known to have any effect against the disease, and (2) to exploit existing leads by systematic modification of the chemical structure of agents that are known to be effective against the disease.

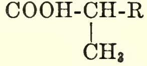
Dr. Leon Schmidt had planned to present evidence from his studies of the effects of alkylating agents on the Walker carcinosarcoma 256, which indicates that these agents vary markedly in their therapeutic efficacies and chemotherapeutic indexes. In his absence I will present similar evidence from our studies of the effects of alkylating agents on the Dunning (IRC 741) leukemia.

The Dunning leukemia is transplanted subcutaneously by implantation of tumor fragments in young, rapidly growing Fischer (line 344) rats. The tumor takes in essentially 100 percent of animals, and kills the animals 12 to 16 days later. Survival is the prime parameter to evaluate therapeutic activity. Drugs are administered daily for 12 days, beginning 24 hours after transplantation of the tumor. Animals surviving 90 days after tumor transplantation without evidence of disease are reinoculated with the tumor. If the tumor takes and kills the animal in the expected time, it is assumed that the disease was cured by treatment.

The dose of each drug is varied over a range that extends from a dose that has no effect on survival to a dose that kills all animals. The total dose of drug in micromoles per kilogram is plotted against increase (over controls) of mean survival time in days, percent of animals cured, or percent of animals killed by the drug (toxic deaths). The minimum total dose required to produce an increase in mean survival time (over controls) of 3 days is determined graphically and recorded as the minimum effective dose (MED). The minimum total dose required to produce 17 percent cures is determined graphically and recorded as the minimum curative dose (MCD). The minimum total dose that would be expected to kill 17 percent of animals is determined graphically and recorded as the minimum lethal dose (MLD). The ratios of $\frac{\mathrm{MLD}}{\mathrm{MED}}$ and $\frac{\mathrm{MLD}}{\mathrm{MCD}}$ are determined and recorded as chemotherapeutic indexes.

Table 1 records the results of a study of the effects, on the Dunning leukemia, of nitrogen mustard and 3 derivatives of nitrogen mustard. Three hundred μmoles of the first compound, nor-nitrogen mustard, are required to produce a minimal effect on the disease. The minimum curative dose is 1200 μmoles and lethal toxicity appears when the total dose is 1300 μmoles. Comparing these results with those of nitrogen mustard, the second compound, one can see that the addition of a methyl group to nor-nitrogen mustard produced a striking increase in therapeutic activity. With nitrogen mustard the MED was 3 μmoles per kg., the MCD was 14 μmoles per kg., and the MLD was 18 μmoles per kg. While this relatively minor change in chemical structure produced a 100-fold increase in chemotherapeutic activity, there was a comparable increase in toxicity; thus the chemotherapeutic indexes are essentially unchanged.

TABLE 1.—Quantitative comparison of chemotherapeutic activity in Fischer rats with the Dunning leukemia. I. Aliphatic analogues of nitrogen mustard

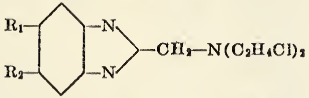
	MED*	MCD*	MLD*	$\frac{\mathrm{MLD}}{\mathrm{MED}}$	$\frac{\mathrm{MLD}}{\mathrm{MCD}}$
$R=N(C_2H_4Cl)_2$					
H-R	300	1, 200	1, 300	4. 3	1. 1
H_3C-R	3	14	18	6. 0	1. 3
COOH-CH_2-R	26	180	205	7. 9	1. 1
 $\mathrm{COOH\text{-}\underset{\underset{\displaystyle CH_3}{\vert}}{CH}\text{-}R}$	40	170	250	6. 3	1. 5

*Total dose μmoles per kg. Drugs were administered in carboxymethyl cellulose (CMC) + saline intraperitoneally daily for 12 days.

The addition of substituents on the methyl group of nitrogen mustard to produce glycine mustard, compound 3, and alanine mustard, compound 4, reduced the chemotherapeutic activity, so that more of the material was required to alter or cure the disease, and since there was a comparable change in toxicity, the chemotherapeutic indexes were not changed appreciably.

Table 2 shows the results of a similar study with a series of benzimidazole mustards. In this system benzimidazole mustard, synthesized and studied previously by Hirschberg, Gellhorn, and Gump, produced minimal therapeutic effect when a total dose of 6.8 μmoles per kg. was administered. The MCD was 76 μmoles per kg. and the MLD 88 μmoles per kg. The chemotherapeutic index for cure was about the same as that for nitrogen mustard.

Table 2.—Quantitative comparison of chemotherapeutic activity in Fischer rats with the Dunning leukemia. II. Analogues of benzimidazole mustard

	R1	R2	MED*	MCD*	MLD*	MLD/MED	MLD/MCD
	—	—	7	75	88	10. 7	1. 2
	Cl	—	75	200	500	6. 7	2. 5
	Cl	Cl	36	—	82	2. 3	—
	CH_3	CH_3	20	80	200	10. 0	2. 5

* Total dose µmoles per kg. Drugs were administered in CMC + saline intraperitoneally daily for 12 days.

The 5 (or 6) chloro derivative of this compound was less active on a molar basis than the parent compound, but the ratio of MLD per MCD was increased significantly, which suggested that this halogenated derivative may be superior to benzimidazole mustard as a therapeutic agent.

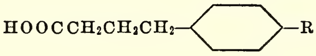
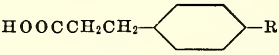
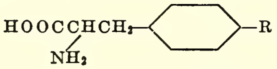
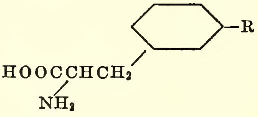
The 5,6 dichloro compound produced highly significant prolongation of life of treated animals, but we have been unable to demonstrate curative activity, and there was an increased toxicity with this compound.

The 5,6-dimethyl benzimidazole derivative proved minimally effective at 20 μmoles per kg., curative activity occurred at 80 μmoles per kg., and the MLD was 200 μmoles per kg. With this compound the chemotherapeutic index based on MCD was more than twice that of benzimidazole mustard.

These results indicate that systematic structural modification of 2-[di-(2-chloroethyl)-aminomethyl]benzimidazole resulted in marked modification of chemotherapeutic activity and toxicity, and produced 2 compounds that are superior chemotherapeutic agents for the Dunning leukemia in Fischer rats. Whether these differences are meaningful in man can be determined only by appropriate human studies.

The results of studies of a series of aromatic alkylating agents are shown in table 3. The first 2 compounds in this table are nitrogen mustard and chlorambucil (CB 1348). The MED of chlorambucil was about 7 times, and the MCD of chlorambucil was about 5 times that of nitrogen mustard, but the MLD of chlorambucil was 14 times that of nitrogen mustard. The chemotherapeutic indexes of chlorambucil were more than twice as great as those of nitrogen mustard. These results indicate that, in the Fischer rat with the Dunning leukemia, chlorambucil is somewhat less potent than nitrogen mustard, but is still highly effective and has a wider margin of safety between the effective and the toxic

TABLE 3.—Quantitative comparison of chemotherapeutic activity in Fischer rats with the Dunning leukemia. III. Aromatic analogues of nitrogen mustard

R= M -N$(CH_2CH_2Cl)_2$		MED*	MCD*	MLD*	$\frac{MLD}{MED}$	$\frac{MLD}{MCD}$
CH_3—R		2.2	9.6	14	6.4	1.4
		14.0	50.0	198	14.0	3.8
		20.0	126.0	180	9.0	1.4
	l	3.2	20.0	70	22.0	3.5
	dl	3.5	15.0	59	17.0	3.9
		(1.5)	(14.0)	(42)	(28.0)	(3.0)

*Total dose μmoles per kg. Drugs were administered in CMC + saline intraperitoneally daily for 12 days.

dose. This is similar to the effects of these 2 agents in humans with chronic lymphatic leukemia and sensitive lymphoid tumors.

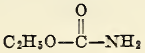
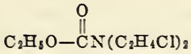
The third compound in this list (CB 1364) differs from chlorambucil by one methyl group in the acid side chain. The chemotherapeutic activity (MED, MCD) was approximately the same as that of chlorambucil, but the toxicity was somewhat greater. Hence the chemotherapeutic indexes of this compound were less favorable than those of chlorambucil.

The addition of an α-amino group to the acid side chain of CB 1364 produces phenylalanine mustard (Melphalan and Sarcolysin). This change produces a striking increase in chemotherapeutic activity because both the MED and MCD are one sixth that of CB 1364. The toxicity of the molecule was also increased, but to a lesser extent. Hence the chemotherapeutic indexes were increased significantly, which resulted in a superior chemotherapeutic agent for the treatment of the Dunning leukemia in Fischer rats. These data suggest that phenylalanine mustard is a chemotherapeutic agent with a potency close to that of nitrogen mustard, but with a margin of safety between efficacy and toxicity that is at least equivalent to that of chlorambucil.

Additional data in table 3 indicate that the *l*-form of phenylalanine mustard is indistinguishable from the racemic mixture (*d*/*l*), and that meta-sarcolysin is also similar. These observations make one doubt that the chemotherapeutic activity of these materials is related to their resemblance to the natural amino acid, phenylalanine. They suggest that a clinical comparison of Sarcolysin and chlorambucil would be of interest.

A comparative study of the chemotherapeutic activity of urethan, urethan mustard (a compound in which nor-nitrogen mustard and urethan are combined in a single molecule), and nor-nitrogen mustard is presented in table 4.

TABLE 4.—Quantitative comparison of chemotherapeutic activity in Fischer rats with the Dunning leukemia. IV. Urethan and urethan mustard

	MED*	MCD*	MLD	$\frac{\text{MLD}}{\text{MED}}$	$\frac{\text{MLD}}{\text{MCD}}$
 $C_2H_5O{-}\overset{\overset{O}{\|}}{C}{-}NH_2$	14, 560	—	63, 000	4. 4	—
 $C_2H_5O{-}\overset{\overset{O}{\|}}{C}N(C_2H_4Cl)_2$	400	—	1, 200	3. 0	—
$HN(C_2H_4Cl)_2$	300	1, 200	1, 300	4. 3	1. 1

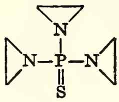
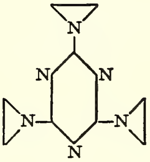
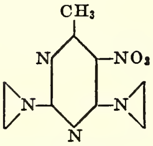
*Total dose μmoles per kg. Drugs were administered in CMC + saline intraperitoneally daily for 12 days.

Urethan has a readily demonstrable effect in suppressing this leukemia, but curative activity has not been demonstrated. This minimum effective dose of urethan is 14,500 μmoles per kg., and the MLD is 63,000 μmoles per kg.

The addition of a bis-β-chloroethyl group to urethan produces a striking increase in chemotherapeutic activity so that the MED is 400 μmoles per kg. (less than 1/10 that of urethan). Toxicity is also increased so that the chemotherapeutic index is unchanged. The MED and MLD of urethan mustard and nor-nitrogen mustard are very similar. This would be expected if urethan mustard were rapidly hydrolyzed *in vivo* to yield nor-nitrogen mustard. We have not yet demonstrated curative activity with urethan mustard, but these studies are incomplete. These observations suggest the possibility that a comparative study of urethan, urethan mustard, and nor-nitrogen mustard in patients with multiple myeloma and chronic leukemia would be of interest.

A comparison of the chemotherapeutic activities of 3 alkylating agents of the ethyleneimine type is shown in table 5. The first compound, commonly known as thio-TEPA, is active and curative. The chemotherapeutic indexes are similar to those of nitrogen mustard (*see* table 1). Triethylene melamine (TEM), which also has 3 ethyleneimine groups, is considerably more active and more toxic, but the chemotherapeutic index based on curative activity is somewhat greater than that of TSPA.

TABLE 5.—Quantitative comparison of chemotherapeutic activity in Fischer rats with the Dunning leukemia. V. Ethyleneimine analogues

	MED*	MCD*	MLD*	MLD/MED	MLD/MCD
	9. 0	25. 0	37. 0	4. 1	1. 4
	1. 5	3. 2	7. 0	4. 7	2. 2
	5. 4	17. 0	45. 0	8. 0	2. 6

*Total dose μmoles per kg. Drugs were administered in CMC + saline intraperitoneally daily for 12 days.

The third compound shown is a pyrimidine derivative with two ethyleneimine groups which was synthesized by Dr. Robert Elderfield and his coworkers. This compound is intermediate in chemotherapeutic activity between thio-TEPA and TEM, but is less toxic than either. Hence its chemotherapeutic indexes are greater, and it is superior to TEM and TSPA as an agent for the treatment of the Dunning leukemia in Fischer rats. Comparative clinical trial of this agent is indicated by these data.

These studies demonstrate quite clearly that all so-called alkylating agents are *not* the same when their chemotherapeutic activity and host toxicity are compared quantitatively in Fischer rats with the Dunning leukemia. Similar results have been obtained by Dr. Leon Schmidt and his coworkers, using the Walker carcinosarcoma 256 and other transplanted tumors, by Dr. Goldin and his group, using Leukemia L1210 in mice, and by others.

It now seems certain that all alkylating agents are not the same in experimental animals with transplanted tumors. It also is clear that systematic modification of the chemical structure of alkylating compounds will produce some dissociation of activity and toxicity and will result in superior chemotherapeutic agents for the treatment of transplantable rodent tumors. This provides us with the basis for believing that further synthesis and testing of agents of this type are indicated and should be one important facet of the Cancer Chemotherapy National Program.

The discovery of better agents for treating transplantable rodent tumors is certainly not our principal objective, but it may be an important first step in achieving our objective of finding better drugs for the treatment of human cancer. The next step in this approach must be to determine whether these differences, which are readily demonstrable in rodents with transplantable tumors, have any correlation or predictive value for the response of any type of human cancer to these agents. Similar quantitative data on the effects of these agents in patients with responsive tumors are urgently needed and are not now available.

Table 6 records the MED, MCD, and MLD, as we determine them in rats with the Dunning leukemia, for 9 alkylating agents that have had relatively extensive human trial. From the published reports of clinical studies with these agents I have estimated, to the best of my ability, the total MED in μmoles per kg. for responsive human tumors, and the total maximum tolerated human dose (MTD) in μmoles per kg. For this purpose I have utilized the most commonly accepted route and regimen for each agent. Recognizing that these figures are merely crude estimates, based on inadequate and variable data, I believe that they have sufficient validity for making a comparison and raising a question.

If one compares the number of μmoles per kg. required to produce cure (MCD) of the Dunning leukemia with the number of μmoles per kg.

TABLE 6.—Comparison of chemotherapeutic activity of 9 alkylating agents in Fischer rats and man

Alkylating agents	Fischer rat			Man	
	MED	MCD	MLD	MED	MTD
HN2	2.2	9.6	14	1.0	2.0
TSPA	9	25	37	5	15
TEM	1.5	3.2	7.0	0.5	2.5
CB 1348	14	50	190	5	40
Sarcolysin	3.2	20	70	6	14
Nitromin	5.0	66	390	15	50
Endoxan	12	80	270	8	50
Mannitol	2.0	50	85	3	27
A-139	1.0	5.0	10	1.5	4.0

that is the approximate MTD in man, it is apparent that we are unable to achieve, with any of these agents, the dose in man that produces cure of the Dunning leukemia. Moreover, the MLD in the rat is considerably greater for each drug than the clinical MTD. This suggests that the susceptible normal tissues of the rat (bone marrow, gastrointestinal, epithelium, etc.) are more resistant to the effects of alkylating agents than are susceptible normal human tissues. If this is true, a search for the cause of this difference would be of importance, and it may be possible that the alkylating agents have the ability to cure susceptible forms of human cancer, if sensitive normal human cells could be protected selectively from their deleterious effects. If we can dissociate chemotherapeutic activity against neoplastic cells from deleterious effect on sensitive normal cells by systematic modification of chemical structure, guided by accurate quantitative biological testing, further study of alkylating agents might yield chemotherapeutic agents of much greater value for the treatment of human cancer.

Dr. Zubrod: The former Director of the National Cancer Institute, Dr. Carl Voegtlin, when presented with a brilliant piece of work by one of the staff would often ask, "What does it have to do with cancer?" This question has been asked, by a number of speakers, in this form: "What do the transplanted rodent tumors have to do with human cancer?" I shall try to answer this question.

The precise quantitative data, which Dr. Jones has shown us, and the work of Dr. Schmidt and of Dr. Goldin are impressive in pointing out that there are marked qualitative and quantitative differences in the activities of various alkylating agents in transplanted rodent tumors. Dr. Baker as a chemist and Dr. Jones as a biologist now want to know whether there exist corresponding qualitative and quantitative differences in the actions of these drugs upon patients' tumors. As a first approach in trying to answer them, I will mention a few notions of my own about animal screens.

I would define a screen as a biochemical, biological, or animal system that will select drugs useful for the control of human disease. As Dr. Skipper pointed out, screens are either semirational or wholly empiric. By rational screen, I mean one in which the etiology is similar in the experimental animal and in the human disease. Pneumococcal infection is a good example. The response of pneumococcal infection to drugs in the mouse predicts reasonably accurately what the drugs will do in patients with pneumococcal disease. With this type of screen, one can rapidly assess new agents and this, of course, has been the history of antibacterial chemotherapy.

A rational screen might also be one in which there existed an identical mechanism, biochemical or physiological, in the screening animal and man. Thus, for example, the action of adrenolytic compounds in the dog will rapidly predict what these compounds will do in adrenal cancer in man.

The empiric screen provides much less direct evidence, and the experience has been everywhere, in all kinds of screening of drugs for human disease, that the empiric screen leads to useful drugs slowly and hesitatingly. The transplanted rodent tumors, we must grant, are empiric screens. They probably are not related etiologically to human cancer, and they probably are not closely related biochemically to human cancer. As with most empiric screens, their great deficiency is that they frequently fail to predict accurately for human disease. Is the failure of the transplanted rodent tumor to predict accurately for human cancer due to the fact that the screen is intrinsically poor, or is it partly due, as Dr. Jones intimated, to lack of knowledge and failure to control the experimental variables in the animal system?

Table 7 shows a list of experimental conditions that are known to have importance in the response of animal tumors to drugs. These relate to the tumor, the host, and the drug. Most of these 10 variables can be manipulated to change markedly the degree of activity of a chemotherapeutic agent upon a transplanted animal tumor. In other words, the drug activity can be shifted at the will of the experimenter in accordance with his particular experimental conditions. In the screening of new drugs for antitumor activity in the transplanted rodent tumor systems, unless the data clearly indicate control over these 10 variables, one will always be uncertain whether "increased activity" represents true enhanced activity or is due to some shift in the pharmacological design. As long as this uncertainty exists it is not possible to answer the chemist and biologist concerning correlation of the screen and the patient, for the correlation can be examined only when it can be based upon the fixed point of true activity in the animal screen. Most animal screening until recent date has not been controlled in this fashion, but the work of Jones, Schmidt, and Goldin has shown how this can be accomplished. We can now begin to frame what might be the steps leading to a study of the correlation of the transplanted rodent tumor screens and cancer in man.

TABLE 7.—Variables influencing activity

Tumor	Host	Drug
Type	Strain	Route
Site	Weight	Vehicle
Inoculum size		Schedule
Type of end point		Eary versus late

Just as it is important to be able to differentiate true antitumor activity of drugs from experimental conditions, it is also essential to be able to make this distinction in patients with cancer. If we assume that it is possible to control the conditions of drug trial in the animal and patient, it then becomes possible to rank a drug in terms of its true antitumor activity in both screen and human tumor. I would propose that this be done and that those animal screens, which rank drugs in the same order as is true in man, be then used to screen for new and more active agents. Although we do not as yet possess sufficient quantitative data either in the transplanted rodent tumors or in patients, let us look at one or two examples from the data at hand.

Table 8 shows such a qualitative comparison for alkylating agents in the rat tumor, Walker carcinosarcoma 256, and in Hodgkin's disease and lymphocytic lymphosarcoma in patients. The rat data were supplied to me by Dr. Leon Schmidt and are true for only a single set of conditions. The patient data are my own estimates, and could unquestionably be improved upon by a direct study. The point I wish to make is that the Walker 256 does not predict very well for Hodgkin's disease but seems to do reasonably well in ranking drugs for their activity in lymphocytic lymphosarcoma. I would suggest then that Walker 256 might not be the best screen for finding more active drugs for Hodgkin's disease.

TABLE 8.—Ranking of alkylating agents in their order of activity for Walker carcinosarcoma 256 in the rat, and for Hodgkin's disease and lymphocytic lymphosarcoma in patients

Walker 256	Hodgkin's disease		Lymphocytic lymphosarcoma	
C*	HN2	0.60†	C	0.5
T	TH	0.40	T	0.3
N	T	0.25	HN2	0.3
TH	C	0.18	TH	0.05
HN2	N	0.10	N	—
M	M	0	M	—

*C = chlorambucil; T = triethylenemelamine; N = Nitromin; TH = thio-TEPA; HN2 = nitrogen mustard; M = Myleran.

† The decimals refer to proportion of patients achieving excellent or good remission.

Table 9 suggests that Lymphosarcoma R2788 (Dunning) is less useful than Lymphoid leukemia L1210 in ranking activity of drugs for acute lymphatic leukemia or choriocarcinoma.

As indicated previously, data are too incomplete to attempt such a correlation at present. These examples are offered solely to illustrate a methodologic approach. The techniques for such a correlation are available, and such a study could put empiric screening on a firm foundation. This could be done only for those tumors in man known to be sensitive to chemotherapeutic agents. It is not suggested necessarily as the best way to screen for drugs for the presently insensitive tumors in man, though this, too, is subject to reappraisal. By the use of such a correlative examination we could quickly answer the questions of the chemist about structure-activity relationships.

We have heard from the chairman that cyclophosphamide (Cytoxan) has shown great activity in some of these quantitative tumor systems. In tables 10 and 11 are preliminary data, attempting to show the correlations

TABLE 9.—Ranking of drugs in order of activity in 2 transplanted rodent tumors, in acute lymphatic leukemia in children, and choriocarcinoma in women

Lymphosarcoma R2788 (Dunning)*	Lymphoid leukemia L1210†	Acute lymphatic leukemia‡	Chorio-carcinoma‡
N	MTX§	6-MP‖	MTX
C	TH	MTX	N
T	6-MP	$0\begin{cases}\text{HN2}\\\text{N}\\\text{TH}\end{cases}$	HN2
TH	T		
HN2	HN2		
MTX	N		
6-MP	C		

*Author's interpretation of data, courtesy Dr. Ralph Jones, Jr.

†Data from Dr. Abraham Goldin.

‡Rankings are author's.

§MTX = methotrexate; ‖6-MP = mercaptopurine. *See* footnote under table 2 for other abbreviations.

among several animal and human tumors. They do not represent a quantitative clinical trial but are simply the observations made in the early part of the pharmacologic work-up. In table 10, cyclophosphamide is seen to have 62 percent of the activity of 6-MP against Adenocarcinoma 755, in terms of prolongation of survival time. Since Adenocarcinoma 755 seems to predict for acute myeloid leukemia, it is interesting to note that cyclophosphamide has induced remissions in this disease. Similarly, cyclophosphamide is as active as methotrexate in L1210, which we hypothesize is the best screen for acute lymphatic leukemia in children. Again, cyclophosphamide has apparently produced good partial remissions in this disease. In table 11 are shown similar data for the lymphomas. You will recall from other speakers that cyclophosphamide was highly active in those tumors we believe predict for the lymphomas. Although one cannot assess the comparative activity of cyclophosphamide from these data, it would seem to bear out partially the predictions of the animal tumors. Knowing that these tumors seem to be quite sensitive to cyclophosphamide, we could now make quantitative comparisons in these specific diseases and see whether the secondary screens have predicted a compound which has a much greater order of activity than nitrogen mustard. Similar studies are in order for phenylalanine mustard, which is the other compound high on the rank order in the quantitative screens.

TABLE 10.—Comparison of results of cyclophosphamide on Adenocarcinoma 755 and Lymphoid leukemia L1210, acute lymphatic leukemia in children, and acute myelocytic leukemia in adults*

	Adeno-carcinoma 755	Lymphoid leukemia L1210	Acute lymphatic leukemia	Acute myelocytic leukemia
6-MP	100	57	36%	20%
MTX	0	100	25%	1/30
Cyclophosphamide	62	97–150	4/36	4/20

*The mouse data were supplied through the kindness of Dr. Abraham Goldin. The numbers represent arbitrary units of activity with 6-MP given a standard ranking of 100 for Adenocarcinoma 755, and MTX for Lymphoid leukemia L1210.

TABLE 11.—Effects of nitrogen mustard or cyclophosphamide on the lymphomas*

	Reticulum-cell sarcoma	Lymphocytic lymphosarcoma	Hodgkin's disease	Giant follicle lymphoblastoma
HN2	1/5	7/22	17/32	—
Cyclophosphamide	5/8	16/26	19/28	3/3

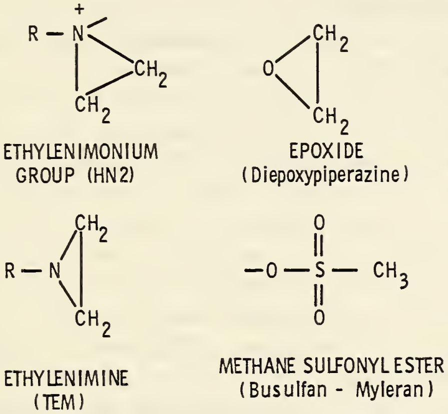
*The figures for the clinical observations are supplied through the courtesy of Dr. Grant Taylor, Southwestern group, Dr. Wayne Rundles, Southeastern group, Dr. John Louis, Midwestern group, Dr. Henry Close, Veterans Administration group, and Dr. Emil Frei, leukemia group B and Eastern group. The numbers represent percentage of patients (or proportion, where few patients have been studied) achieving complete or extensive partial remission.

Let me return to the original question of what the transplanted rodent tumors are telling us about alkylating agents in man. The purely qualitative studies of the past have left a vague impression that all alkylating agents are much alike. The application of quantitative techniques to animal testing now tells us clearly that there are great differences in the activity of alkylating agents in the transplanted tumors. Similar quantitative data in man have yet to be collected, but, to some of us, the need for applying the results of these new quantitative techniques to man is an essential step in deciding upon the predictability value of the transplanted rodent tumors and their future value in finding drugs to control human cancer.

Dr. Karnofsky: My comments will represent a minority report. In 1957 the New York Academy of Sciences held a conference on Comparative Clinical and Biological Effects of Alkylating Agents (*1*). One hundred and ten people presented papers at this conference. Dr. Alexander Haddow, Director of the Chester Beatty Research Institute in London, whose staff has made many important contributions to our understanding of the mechanism of action of the polyfunctional alkylating agents, concluded the conference by stating: "A main purpose [of this conference] has been to determine whether any of the alkylating agents have characteristic qualities or chemotherapeutic advantages that distinguish them from others. I am forced to conclude that there is no compelling evidence that this is so." (*2*).

The question of whether the polyfunctional alkylating agents can be altered so that they will have a different spectrum of therapeutic activity or a wider therapeutic index is under continuing study. We may ask if any information has accumulated since 1957 to alter Dr. Haddow's unchallenged conclusion.

Chemically speaking, there are 4 major types of polyfunctional alkylating agents (text-fig. 1). A number of variations in structure have been produced by attaching carrier substances to each of these groups. These carriers have altered the properties of the polyfunctional alkylating agents in such matters as solubility, reactivity at different *p*H's, and acute pharmacologic effects. When given to man, they have exhibited different activities in relation to tolerance by the oral route, efficiency of absorption from the digestive tract, and emetic, cholinergic and psychogenic effects. Alterations in structure have made it easier to use specific polyfunctional alkylating agents for certain situations and thus widen their clinical applicability. It is hoped, of course, that the ease in modifying the acute pharmacological properties of these drugs can be matched by improvement in their therapeutic activity.



TEXT-FIGURE 1.—Polyfunctional alkylating agents; reactive groups.

There is good evidence, however, that the effective polyfunctional alkylating agents, irrespective of their prosthetic groups, interfere with proliferating cells because of specific effects on deoxyribonucleic acid (DNA). This action is manifested in animals by damage to proliferating tissues, such as the bone marrow, intestinal mucosa, and germinal epithelium, and to transplantable tumors. This mechanism of cytotoxic activity is believed to transcend in importance injury to other components of the cell. If we assume that the alkylating agents have a common mechanism of action in damaging proliferating normal and neoplastic cells, it would seem unreasonable to hope that a new derivative could be produced that would be more specific against neoplastic cells in general or certain types of cancer specifically, as compared to their effects on normal proliferating tissues. If dissociation between toxicity to normal tissue and antineoplastic activity could be clearly proved, it would indicate a difference between normal and neoplastic cells, and provide a wedge toward enhancing the specific anticancer activity of the polyfunctional alkylating agents. Such clear evidence has not yet been produced. Animal data do not indicate any qualitative differences in the mechanism of action of these compounds, and quantitative differences may possibly be explained by the chemical behavior and metabolism of these drugs. The surprising thing is that, despite great variations in structure, these agents are very similar in their cytotoxic actions when given in adequate doses.

There is evidence, nevertheless, that certain transplantable tumors in mice and rats show quantitative differences in their response to different polyfunctional agents. These differences have been discussed by Dr. Jones and Dr. Zubrod. The data obtained in rather esoteric and complex systems, rendered familiar but no less bizarre by common usage, cannot be used to support interpretations of clinical results, particularly in studies comparing the activities of related drugs. Human data must stand on its own feet, separate and distinct from mouse results that may have stimulated the clinical study.

An important point that might be discussed is what we are accomplishing with these agents in the treatment of human cancer. The assumption is that these drugs in the mouse or the rat are destroying tumor cells, but actually there is reasonable evidence that these agents in certain types of human cancer are acting by different mechanisms other than a direct destruction of neoplastic cells. For example, in Hodgkin's disease, when one produces tumor regression or detoxification of the patient, such as a fall in fever or relief of itching, this is not necessarily associated with an obvious destructive action on the neoplastic cells themselves. We may be modifying some reaction on the part of the host to the presence of this tumor. We should carefully review why we are so enamored with mouse tumors as being closely correlated with human cancer. It is conceivable that the intestinal tract of the mouse may be a better indicator of compounds, which have specific effects on human cancer, than transplantable tumors. We know, in regard to the polyfunctional alkylating agents, that the intestinal mucosa of the mouse is sensitive to their cytotoxic action, whereas the human digestive tract is relatively resistant. Perhaps drugs that damage the mouse intestine may serve as well or better than transplantable tumors in predicting those compounds which will have antitumor activity in man. This suggestion may be too radical to be seriously considered, but I would like to emphasize that we are using tumors in mice as a critical and perhaps basic yardstick for the types of agents and their derivatives that are to be tested in man.

The comparative evaluation of polyfunctional alkylating agents active against animal tumors and in various forms of cancer has been discussed many times, and it is a difficult problem. This is particularly true because these drugs usually have a negligible, transient, and inconstant therapeutic effect on many types of neoplastic disease.

In fact, I can only ask Dr. Zubrod again exactly how he plans to carry out quantitative studies on neoplastic disease in man.

One would have to change the nature of cancer in man to obtain the kind of precise data for these weak agents that would permit one to grade them as to comparative therapeutic value. We know that the polyfunctional alkylating agents rarely produce any prolongation in life, and what we measure are transient regressions in tumor size. The radiotherapists long ago learned to distinguish between radiosensitivity, which is the transient regression of a mass, and radiocurability, which means a substantial and prolonged effect in a local cancer. When therapeutic differences between the two drugs are admittedly not great, if they exist at all, very refined criteria of evaluation under carefully controlled conditions are required to obtain any evidence as to their comparative activity. These comparative studies must be conducted by giving equivalent amounts of a drug over the same period and quantitating the clinical benefit produced at comparable degrees of systemic toxicity. The same types of disease in patients with the same patterns of evolution of the disease and in the same stages in each pattern should be compared for meaningful results.

Comparative clinical studies with drugs, with the use of arbitrarily selected doses, are open to serious question. The therapeutic index is narrow for the great majority of anticancer agents and is nonexistent in many cases, and the judgment of the physician in giving the optimal dose is essential to obtain useful comparative data.

Most clinical studies have not supported the suggestion that the polyfunctional alkylating agents are qualitatively different in their therapeutic effectiveness. Quantitative differences have been described, but these may be related, under identical conditions of testing, to differences in dosage sequence and degrees of toxicity produced. Perhaps the most striking derivative, already mentioned, is Cytoxan, which apparently differs from some of the other alkylating agents in that extravasation causes no local damage, it produces leukopenia without thrombocytopenia, and is unusually active in causing alopecia. This agent, however, does not appear to have a unique therapeutic spectrum of activity, though there is perhaps a diminished hazard in the lesser tendency to thrombocytopenia.

All the alkylating agents studied thus far seem to be useful in similar situations. They will temporarily suppress manifestations of certain forms of cancer, but they have not appreciably prolonged life in the vast majority of patients.

In the acute leukemias, the alkylating agents will reduce the white count, cause symptomatic benefit in such matters as bone pain, or decrease local infiltrations, but hematological remissions are extremely rare, if they occur.

In chronic myelocytic leukemia, the alkylating agents will regularly suppress the disease, but there is no evidence that they have prolonged life. In chronic lymphatic leukemia, these agents are effective and may cause a profound therapeutic response in about 25 percent of the patients; this response may be perhaps related to prolongation of life. These patients, incidentally, are sometimes unusually susceptible to conventional doses of any one of the available polyfunctional alkylating agents.

In Hodgkin's disease, the alkylating agents can cause lymph-node regression and relieve fever and other systemic manifestations of the disease for a number of weeks or months, depending upon the inherent activity of the disease. In lymphosarcoma, certain patients may respond just as sensitively as some with chronic lymphatic leukemia, whereas others are unusually resistant.

In mycosis fungoides, as in lymphosarcoma, there are occasional excellent responses and some very transient ones.

In lung cancer, nitrogen mustard has produced subjective improvement and objective tumor regression in about 30 percent of the patients. The responses have been brief.

In ovarian cancer, a number of alkylating agents have been effective, and about 30 percent of the patients have been temporarily benefited.

In carcinoma of the breast, nitrogen mustard may briefly relieve certain complications such as symptoms or signs due to cerebral or pulmonary metastases, in rare situations, and cause regression of palpable disease.

In testicular tumors, tumor regression has been reported in a few patients, particularly those with pulmonary metastases.

Among the miscellaneous types of tumors, a consistent incidence of improvement has not been reported, but benefit has been observed occasionally in multiple myeloma, melanoma, carcinoma of the cervix, Kaposi's disease, spindle-cell sarcoma, Wilms' tumor, choriocarcinoma, and in others.

In conclusion, there appears to be no compelling evidence that the polyfunctional alkylating agents differ qualitatively in their chemotherapeutic activity in man. None prolong life appreciably in any form of neoplastic disease, their effects are essentially similar in various clinical situations, and they all produce bone marrow depression to some degree at therapeutic doses, and to a severe degree, at excessive doses. Yet there are some differences that enhance their range of usefulness in various clinical situations. Gellhorn has compared the situation as far as the selective effects of the alkylating agents against cancer are concerned to the status of the numerous digitalis preparations in the management of heart failure (*3*).

While I would like to look forward hopefully to more specific polyfunctional alkylating agents, clinical evidence forces me to conclude that this is a forlorn hope. At present, if one has nitrogen mustard, triethylenemelamine (TEM), chlorambucil, and Myleran at his disposal, he can manage all clinical situations responsive to the systemic administration of an alkylating agent. For the future, therefore, these agents will continue to be used as indicated until something better comes along.

Dr. Baker stated that we are only at the frontier as far as the alkylating agents are concerned and that the justification for this conclusion was to provoke thought. I want to take refuge behind the same hedge. If Dr. Baker or Dr. Bergel or anyone in the future discovers specific anticancer alkylating agents, I will insist that my statements were merely intended to provoke thought.

REFERENCES

(*1*) Comparative clinical and biological effects of alkylating agents. Ann. New York Acad. Sc. 68: 657–1266, 1958.
(*2*) Haddow, A.: *Ibid:* 1258–1260.
(*3*) Gellhorn, A.: *Ibid:* 1254–1257.

Dr. Baker: I talked earlier about the two factors necessary for the effectiveness of a compound in the tumor-host-drug relationship: (1) The compound obviously should be toxic to the tumor. (2) It should be selectively toxic. Dr. Jones believes strongly that his data show differences in alkylating agents. I would like to go further and say that it is possible to get quite different tumor spectra, depending upon the alkylating agent, and likely this difference is tied in with the metabolism of the carrier group, either by activation, as Danielli proposes, or by selective detoxification, as I discussed earlier.

Dr. Bergel recently gave us an interesting seminar about some of his work. By constructing peptides of phenylalanine mustard, he was able to demonstrate that the effectiveness, as measured by chemotherapeutic index, on the Walker carcinosarcoma 256 in the rat was greatly increased by a peptide between phenylalanine and phenylalanine mustard. In contrast, the effect on Sarcoma 180 in the mouse was reduced by this peptide.

In 1958 Larionov of the Soviet Union reported at the Pan-Asiatic Cancer Conference that if one made peptides of phenylalanine mustard, one was able to vary the tumor spectrum in man, which made it more or less effective against one or another tumor.

There must be some difference in the way the tumor handles the drug as far as these agents are concerned. The needed alkylating agents, as I mentioned earlier, are becoming much more difficult to synthesize, but much more worthwhile to make. It is within the realm of possibility that by the exploitation of an enzyme system present in normal cells and not in a tumor, one should be able to devise an alkylating agent to take advantage of the difference. Also, if there is an enzyme system in the tumor but in lower concentration in the normal cells, this difference may be exploitable, as represented by Cytoxan. Maybe we can take advantage of these differences, regardless of the fact that all alkylating agents may or may not operate on deoxyribonucleic acid (DNA) as the central point of attack.

My personal feeling as a chemist is that additional work in the alkylating agent area would be useful. I posed this to Dr. Bergel and he gave me a happy answer. He thought that at this stage of the game about 30 percent of the effort of a broad program into synthesis of alkylating agents would be a reasonable amount. I was pleased about this, since our program at Stanford Research Institute is about 30 percent in alkylating agents, about 30 percent in antimetabolites of the classical reversible or nonclassical irreversible type, 30 percent in nucleosides, and the remainder in miscellaneous compounds. We should not differentiate quite so much between antimetabolites and alkylating agents. We still have to take advantage of enzyme differences, and the mechanistic approaches to antimetabolites and alkylating agents will converge.

Dr. Zubrod: Dr. Karnofsky and I are old friends and I have great respect for his advice and opinions. We have frequently been on panels together and usually we are on opposite sides of the table, but I can agree completely with Dr. Karnofsky that we both doubt seriously the ability of the transplanted animal tumor to predict for all cancer in man.

Are the transplanted rodent tumors predicting something worthwhile for man or are they not? If they are not, let us stop using them and go on to something better. My own feeling about transplanted animal tumors is that perhaps they are predicting for a half dozen different diseases that we include in this term "cancer." There are probably dozens of other types of diseases encompassed in this general term of cancer for which the transplanted animal tumors do not predict. For these we perhaps will need entirely different systems, such as Dr. Stock described: the virus-induced tumors, the carcinogen-induced tumors, the radiation-induced tumors, etc., to find active materials.

For the leukemia and the lymphomas, we are in the position of being able to settle the usefulness of the transplanted rodent tumors by either keeping them, discarding them, or saving whatever is truly useful. There is one difference that I have with Dr. Karnofsky. He is looking for a new alkylating agent with qualitative differences, either in the experimental tumor or in man. What I am looking for and what I think Dr. Jones and Dr. Baker are asking on the part of the pharmacologists and the chemists is for an accurate assessment of quantitative differences. Would it not be important if we had an alkylating agent which had the same qualitative effects as nitrogen mustard but which was a 100-fold more active and much less toxic than nitrogen mustard?

Dr. Jones: We are highly honored to have with us from Britain one of the men who has led this field of research over a number of years. I wonder if Dr. Bergel would like to comment on this general topic.

Dr. Bergel: It is very kind of you to ask me to take this special position, but I only want to add a footnote to what Dr. Baker said in his generous way. I think I agree with Dr. Zubrod that in the field of alkylating agents, and we must remember that there are other fields still largely intact, we are looking for improved quantitative relationships, particularly in man. As a chemist I can only say that I am not crossing swords with Dr. Karnofsky about the clinical side, but only wish to tell him and his colleagues that in phenylalanine mustard, either Melphalan or Sarcolysin, we have been anxious to achieve a better therapeutic ratio with some of its derivatives. Larionov in Russia has done the same thing. We have made peptides different from those in Russia and in the animal we think we have achieved an increase of the therapeutic ratio from about 8 to 9 with the actual phenylalanine mustard, to over 20 with the peptide Dr. Baker mentioned. Moreover, we have made tripeptides and are making tetrapeptides and hope to produce a kind of antibiotic satellite which, while still carrying the alkylating group, might in itself have a greater specificity inside the tissue. We have started to put alkylating groups in a latent form on proteins and hope to achieve the same thing when our techniques are right with purified, tumor-specific antiserums. We hope that the alkylating agent, placed on these proteins, might return like a homing pigeon to the actual cell from which the antigen derived. These are the lines along which I think 30 percent of total activity should be directed.

Dr. Chapman: I should like to direct attention to another aspect of the alkylating problem. Although we do not have definitive evidence, it is usually assumed that the bis-β-chloroethyl alkylating agents or the ethyleneimine alkylating agents are chemical identities, carrying on alkylation about equally with the various groups when they are rated for the effectiveness of interactions. I think it is possible to show that the different kinds of alkylating agents, when modified by the kind of groups they carry, not only react very differently chemically but also select targets chemically. For instance, chloroquine mustard may select one type of target, while L-phenylalanine mustard selects another. The point I wish to make, then, is that when you look at an alkylating agent you have a specific active end which can be differentiated chemically and you have a specific carrier end which can be differentiated physiologically. Now if these two things can be put together, it may be possible to derive agents that more or less pinpoint their type of action. All these things could not be assumed automatically to give beneficial clinical results, but, until the mechanisms underlying these actions are known, it is impossible to conclude that they are going to act the same way under all circumstances.

Dr. Baker: I would like to make a comment about the statement that 600 alkylating agents are a lot of compounds. If one looks at the classes of carrier groups that have been investigated, one realizes that many classes of difficultly synthesized compounds have been poorly exploited; in fact, little work has yet been done on substrate type carriers that can be metabolized. We have a great deal of synthetic work yet to be done to try to take advantage of a biochemical lesion characteristic of a given tumor, but not characteristic of all tumors.

Investigations in Acute Leukemia[1]

Chairman: **DR. JOSEPH H. BURCHENAL,** *Memorial Hospital, New York, New York*

Members of Panel:

Dr. Ruth Heyn, *University of Michigan, Ann Arbor, Michigan*
Dr. W. W. Sutow, *M. D. Anderson Hospital, Houston, Texas*
Dr. Emil J. Freireich, *National Cancer Institute, Bethesda, Maryland*
Dr. Richard Whittington, *Veterans Administration Hospital, Cleveland, Ohio*
Dr. John Louis, *University of Illinois, Urbana, Illinois*

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[1] Presented at conference on Experimental Clinical Cancer Chemotherapy, Washington, D.C., November 11, 1959.

Dr. Burchenal: Acute leukemia is one of the neoplastic diseases in which it is easy to evaluate chemotherapy, in contrast to some of the solid tumors, particularly the deep solid tumors, in which it is difficult to quantitate the progress of the treatment. Since 1947 and 1948, when Dr. Farber demonstrated the ability of the folic acid antagonists to produce remissions in this disease, we have been convinced that new chemotherapeutic agents should be given a trial in the acute leukemias.

The fact that in leukemia a white blood count and differential can be studied 3 times a day if necessary, and that marrows can be studied once a week or once a day if indicated, gives precise methods for evaluating the action of the drug. It is much the same situation as in pernicious anemia. The statement has been made many times that the control of pernicious anemia would not have been discovered if it had not been for the reticulocyte count, which gave a precise method of evaluating therapy.

On the other hand, acute leukemia is not as easy for the investigator as chronic granulocytic leukemia, for instance, because the latter is a less rapidly progressing disease. It is possible with this disease to test a negative compound for 2 or 3 weeks or a month without harming the patient. However, a negative compound tried in acute leukemia for a month might well make it impossible to put the patient in remission, even with steroids.

In the acute leukemias we have opportunities for 2 types of studies. We have 3 different classes of agents: the folic acid antagonists, the purine antagonists, and the steroids, which will produce remissions in a reasonable percentage of patients, particularly children. Generally speaking, we cannot try a new agent on a patient who has not been given some therapy with these agents. We can, however, compare new agents of a type that has been shown to have an effect on the disease. For instance, we can compare 2 folic acid antagonists on a type-3 randomized study or we can compare 2 or 3 purine antagonists simultaneously.

In patients who have become resistant to the folic acid antagonists and resistant to the purine antagonists and are still in fairly good shape, there is a wide field for studying new compounds in the phase-1 or phase-1½ type of studies in which the dosage is known, but its effectiveness against leukemia is not known.

There is the need among the different groups for uniform criteria of remissions, of a complete remission, etc., so that we are all talking the same language. These groups have been at work for some time, though they have not all reached uniform criteria. In many cases, it may be better to work out slightly different criteria for a period to see how they fit the studies. When the criteria committee originally assembled the criteria for remissions in acute leukemia, they did it without benefit of experience in working out a cooperative project over a long period on a large number of cases. For that reason, in practice in the cooperating groups, certain changes in the criteria have been necessary. I hope eventually, however, that the criteria will all be the same, particularly those for a complete remission.

When new agents are being studied, particularly in critically ill patients, oftentimes there is confusion. So many situations, in which radiation and bone marrow transfusion have been given or a new agent has been tried, have been confused because the patients were also on steroids at the time, and it was impossible to say that the steroids had not produced the remission that was attributed to the new form of therapy.

Dr. Heyn: I would like to present a brief summary of the activities of the leukemia chemotherapy cooperative study group A. This group was organized in the latter part of 1955 to evaluate new agents in the treatment of acute leukemia in children. It was initially comprised of investigators from 11 institutions and was organized under the guidance of the clinical studies' panel of the Cancer Chemotherapy National Service Center.

In the 47 months since this group was organized, a total of 938 children with acute leukemia have been seen and, of this total, 654 children have been put on a study program. The patients used had been previously untreated and were assigned a study drug by the process of random selection.

Tables 1 and 2 summarize the studies that have been undertaken by our group to date. The first study evaluated the use of 6-mercaptopurine (6-MP) alone compared with the combination of 6-MP and azaserine. Of the 250 patients seen by the group during a 15-month period, 168 were put on a study drug, and, of these, 125 were ultimately available for evaluation.

TABLE 1.—Summary of drug evaluation studies

Study	Months	Total seen	Put on study	Evaluated
6-MP–azaserine	15	250	168	125
6-MP (2.5 mg./kg./day)			100	
6-MP 6.6 (mg./kg./day)	26	540	97	—
6-MP-DON*			101	
Post steroids				
6-MP	26	62	30	
6-MP–DON			32	

*DON = norleucine, 6-diazo-5-oxo (Parke, Davis & Company).

The second study was continued for 26 months, with 6-MP used in two different dosage schedules, one giving 2.5 mg. per kg. per day and the other 6.6 mg. per kg. per day. The third group was the combination of 6-MP with DON. There were 298 children in one of these prorgams, with the distribution given in table 1. The final evaluation of these results

TABLE 2.—Comparison studies of 6-MP, TG, TGR, and FUDR

Study	Months	Total seen	Put on study	Evaluated
6-MP	In progress	86	30	—
TG*			28	
TGR†	7		28	
FUDR‡	In progress		40	
Total	47	938	654	

*TG = thioguanine.
†TGR = thioguanosine.
‡FUDR = 2′-deoxy-5-fluorouridine.

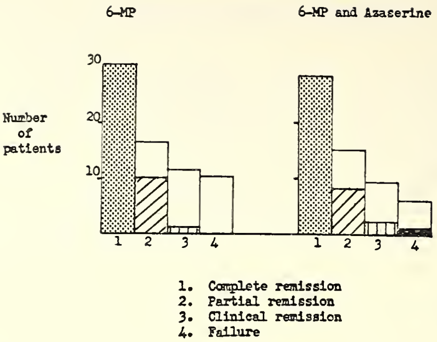
has not as yet been completed. In both studies it is apparent that a large number of patients were either not available for study to begin with or were lost to study. One of the large waste groups was that including children who had already been treated with steroids when they were first seen. One attempt to salvage some of this group was to run a parallel series of the study drugs in children who had been on steroids but were at this point in a state of relapse. In this way 62 additional patients were utilized.

In May, 1959, we began our third study to compare 6-MP, thioguanine, and thioguanosine. To date, 86 patients have been given a study drug with the distribution as shown in table 2. Thirty have received 6-MP, 28 thioguanine, and 28 thioguanosine. It is too early for us to have any data accumulated about these patients. In addition to these studies, we have made a further attempt to salvage patient material by using other new but less promising compounds on a smaller scale in the drug-resistant group. We have 40 patients who have been given FUDR intravenously to date. Miracil D or Lucanthone hydrochloride is another such compound that was used in this drug-resistant group.

Now I would like to return to the first study and its results. The impetus for our initial study, as with many of the others, was based primarily on animal work. Azaserine was shown by the group at Memorial Center to be relatively ineffective when used alone in the treatment of acute leukemia in children. However, a synergistic effect against transplanted mouse L1210 leukemia and mouse Sarcoma 180 was shown when azaserine was combined with 6-mercaptopurine.

Text-figure 1 represents a graphic analysis of the results of this first study. The criteria that we used for evaluation were those that were originally set up by the clinical studies' panel for acute leukemia and which were reported in *Blood* in 1956, by Bisel. Since that time we have applied a different criteria to the subsequent studies which were devised by our study group cooperatively.

As is indicated in text-figure 1, no difference could be shown either in the number of remissions we obtained nor in the duration of those remissions in the two groups. The shaded areas in these groups represent



TEXT-FIGURE 1.—Analysis of the results of treatment of 125 patients on 2 drug programs. *Shaded area* gives the number of "one" marrows seen in each group.

the number of complete marrow remissions that were obtained in each of the remission groups.

Table 3 gives the same results but on a percentage basis, showing the percentage in each group who obtained complete, partial, and clinical remissions, and the percentage of children who represented failures. There actually is no statistical difference between these two groups. These results indicate again that work in animal tumors does not always carry over to clinical trial.

TABLE 3.—Percentage of complete, partial, clinical, and no remission of patients on 2 drug programs

Remission	6-MP (*percent*)	6-MP and azaserine (*percent*)
Complete	44. 8	48. 3
Partial	23. 9	25. 9
Clinical	16. 4	15. 5
Failure	14. 9	10. 3

When we began to use the original evaluation criteria we realized that certain features were not entirely applicable to children. A few areas we considered changing were those relating to peripheral blood counts, for example, where total number of lymphocytes was not great enough to apply to the normal child and where the percentage of lymphocytes in the bone marrow likewise was too low to include many normal children. Another problem we found was in the application of the criteria to physical findings. For example, the size of liver and spleen in young children was taken into account, and our new criteria have made amends for this. We also changed the criteria to give a different rating system primarily in the physical findings, and have applied some simplifying physical measurements.

Dr. Sutow: The Southwest cancer chemotherapy study group is one of the younger cooperative groups. It has, at the present time, 8 participating members in the acute leukemia study. These include Baylor, Arkansas, Oklahoma, and Tulane Medical Schools; Lackland Air Force Base Hospital in San Antonio; and the 3 branches of the University of Texas: the Medical Branch at Galveston, Southwestern at Dallas, and M. D. Anderson Hospital and Tumor Institute in Houston.

The first study undertaken by the group was the evaluation of the comparative effectiveness of hydrocortisone versus ACTH used alone and in sequence with 6-mercaptopurine in the treatment of acute leukemia in children. This was activated in July, 1958. Since that time we have registered 124 children with acute leukemia. Of these, however, only 64 were considered eligible for the definitive study. This loss in patient load was disappointing because one of the reasons steroid was selected in the protocol was that many of the children, at the time they were first seen clinically, appeared to need steroid therapy. It is too early to present any meaningful tabulation of the results of the cooperative study.

The patients who have completed the protocol study or who have been considered ineligible for the study have been treated with Cytoxan. The interesting preliminary data on these children are presented for two reasons: (1) The patients on whom Cytoxan was used had already been treated with the standard drugs. They had become nonresponsive or resistant to all these agents. (2) The responses we had seen after treatment with Cytoxan were qualitatively quite good and unexpectedly frequent. It is possible, of course, that there has been a fortuitous concentration of favorable cases at this time. Further experience may show that our present enthusiasm, though it is rather cautious, may have been a little premature. Perhaps Doctor Freireich will comment later about the experience of leukemia group B with this particular compound, which will provide a much more realistic perspective concerning the potential usefulness of Cytoxan in the treatment of acute leukemia.

We have given 18 courses of Cytoxan to 11 children with acute leukemia. Table 4 summarizes the data.

TABLE 4.—Responses to Cytoxan therapy in children with acute leukemia

Dose of Cytoxan (*mg./kg. of body weight*)	Response (*number of patients*)		
	Good	Partial	None
Oral			
5. 0	1	—	1
2. 0	1	—	5
Intravenous			
5. 0	—	1	1
7. 5	—	4	3
20. 0	—	1	—
Total	2	6	10

The doses of Cytoxan are in mg. per kg. by mouth and intravenously. Two children received doses of 5 mg. per kg. by mouth. Six children were treated on the continuous dose of 2 mg. per kg. Two children received intravenous doses of 5 mg. per kg. over a period of 4 to 7 days. A group of 7 patients received 7.5 mg. per kg. of body weight intravenously over a period of 3 to 7 days, and 1 patient received 20 mg. per kg. for 3 days. The evaluation of responses here follows closely the criteria used in leukemia group A and our group. In complete remissions, the children had normal bone marrow, physical findings, peripheral blood, and activity ratings. Under partial remissions are listed those who did not quite achieve normality though there were substantial degrees of improvement.

In our study there were 2 complete remissions, 6 partial remissions, and 10 failures. Five of these children received more than 1 course of therapy and in 2 we were able to obtain a second partial remission. Of these partial remissions, 2 were actually almost complete. The bone marrow studies were technically inadequate for evaluation and for that reason they were placed under the partial remission category.

The duration of response varied (table 5). In general it was rather short, 6 to 13 weeks. One patient is still in remission at the end of 6 weeks, 1 is in partial remission at the end of 3 weeks, and 1 has just gone into partial remission. We do not know whether he will improve still further or how long he will be in remission.

TABLE 5.—Duration of response to Cytoxan

Remission	Number of patients	Duration (*weeks*)
Complete	1	6+
	1	13
Good	1	3
	1	8
Partial	1	2
	1	3+
	1	5
	1	?

In those who have had favorable responses, improvement occurred early, within 4 to 7 days after start of therapy. In some patients, when it was evident that response was not occurring or was rather slow in appearing, increase in dosage brought about improvements. The drop in the white blood count when it was high, the decrease in size of the organs when they were large, and a decrease in percentage of blasts were rather rapid in some instances.

Alopecia and leukopenia occurred, as reported by others. Of more concern were the occurrence terminally of jaundice in 2 children and evidence of renal failure in 2 others. The causal relationship of the drug administration to these disturbances, however, was not completely clear. Of the drugs that we used in children who had reached this stage in acute leukemia, Cytoxan has given the best performance. We have modified the doses slightly. We are now giving 5 mg. per kg. intravenously for 10 days or until the white blood count drops to 1,500. After the count has stabilized, an oral maintenance schedule at 5 mg. per kg. is started.

The Southwest cancer chemotherapy study group's formal protocol study prescribes steroids as initial therapy. This permits accumulation of ancillary data that may be associated with hormone therapy. Along this line, it may be interesting to present some of our data that touch upon the comments made by Dr. Gehan, who listed some 17 variables of possible prognostic value in predicting responses to therapy (p. 51). These were based on the leukemia group B study. It would be desirable in these leukemia cases, if we could predict with a reasonably practical accuracy the degree of response to any form of therapy. Keeping a child for more than 4 to 8 weeks on investigative therapy, when the disease is advanced, may be a rather precarious undertaking.

Of the 17 variables, Dr. Gehan concluded that age and the type of leukemia rank highest as determinants of predictability regarding response. If children only are studied, the age factor can be eliminated. That leaves the type of leukemia as one of the important considerations.

In 1954 and again in 1959, investigators in Dr. Wintrobe's laboratory reported that the effectiveness of hormone therapy in children with acute leukemia was excellent in the lymphoblastic type, and unsatisfactory in the myeloblastic type. The data accumulated by our group seem uniquely suited to test the degree to which predictions of this sort might be made. We recognized, of course, that there exists today a great lack of agreement, often vociferously expressed, concerning the ability or inability morphologically to classify acute leukemia in children.

The bone marrow smears collected at headquarters from all members of our group were studied by one member of the acute leukemia evaluation committee. Without any clinical information, he divided the slides into 2 categories: those showing the morphologic characteristics associated with myeloblastic leukemia and those not showing the criteria. These criteria were identical with those used by Dr. Wintrobe's group. The clinical records were examined separately by another investigator, a pediatrician. Children were divided into 2 categories again: those who had a favorable response to steroid therapy and those who did not. For the purposes of this study, a favorable response was defined as the attainment of an A-1 or a normal bone marrow.

The observed clinical response in each child was then compared with the predicted response. Data became available on 47 patients. These are summarized in table 6.

Table 6 indicates a favorable correlation between prediction and observation. This is in the right direction of positive predictive ability.

From the clinical point of view, the group that did not respond is the most important. Of the 14 predicted as not responding, 10, in fact, did not. Statistically, this degree of predictability is very significant. The estimate of the clinical usefulness of this type of prediction needs accumulation of more data.

TABLE 6.—Correlation of responses, predicted on basis of morphologic category, with observed responses to steroid therapy

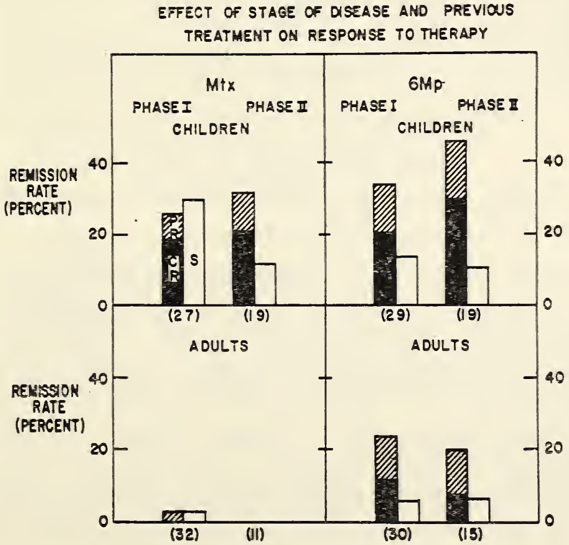
	Predicted responses	
Observed responses	Beneficial	Not beneficial
Beneficial	24	4
Not beneficial	9	10

Dr. Freireich: As Dr. Burchenal emphasized, the chemotherapist treating human acute leukemia is fortunate in his ability to quantitate the responsiveness of this disease to treatment. As mentioned, we are also fortunate that there are agents which are clearly effective in producing not only remission but prolonging the survival of these patients. However, we do not have to resort to survival as the only measure of drug effectiveness. We have the advantage of being able to use the occurrence of remission as an indication of a drug's effectiveness. This is both sensitive and efficient. It gives us rapid answers and it allows us to use many drugs in a single patient for this kind of quantitative information.

On the other hand, the fact that we do have effective chemotherapy for patients with acute leukemia is also a disadvantage, because these drugs are not 100 percent effective and, for those in whom it is not effective, treatment represents a risk to their ability to survive to the point where newer agents can be studied. The treatment is good enough so that most of us would not withhold this therapy and study new agents in untreated patients, even if, by using these agents, we lose a significant portion of the population of this disease for the study of new agents and the discovery of new areas of activity that may become more effective drugs. This is the problem that I would like to discuss. We are usually faced with the prospect of studying these new drugs in very sick patients with advanced disease, and one wonders whether this is a fair trial of any new agent. Are we really studying its effect? Could we, in fact, detect a drug with the order of activity of amethopterin in such patients, if we had this opportunity again?

The acute leukemia cooperative group B has approached this problem by studying the conditioning effect of treatment with one antimetabolite on the responsiveness to another. I am sure you are familiar with the work of Dr. Lloyd Law, and others, which has shown that animal tumors resistant to 6-mercaptopurine or to azaserine show enhanced sensitivity to the antifolics. Our study was designed to determine whether this particular phenomenon occurs in man. Our patients were assigned to two treatment groups, one receiving 6-MP as initial therapy and the other amethopterin as initial therapy. Then, after what we defined as an adequate course of treatment, when these patients were either not responsive to therapy or had remitted and then relapsed on therapy, in other words, were resistant to the first agent, they were switched to the second agent. Thus we had an opportunity to examine the effect of a drug when given as initial treatment to an untreated patient and when given as the second phase of therapy to a patient who had previously received the other drug. The data in text-figure 2 represent a preliminary analysis of the first 200 patients studied.

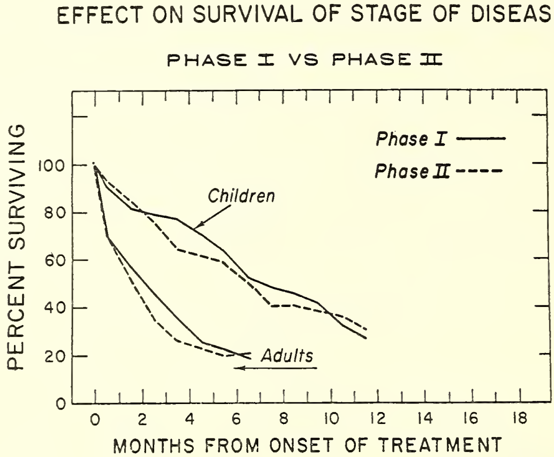
These data are arranged to show the response to amethopterin and 6-MP, in phase I and phase II. Phase I refers to the initial therapy in an untreated patient and phase II refers to the treatment initiated after the patient has either failed to respond to, or is clearly resistant to, treatment with the first agent. If we start on the left side with amethopterin (MTX), amethopterin in phase I was initial therapy, and, in phase II, all patients had received 6-MP as the first phase of treatment. Similarly with 6-MP on the right side of text-figure 2, phase-I treatment was untreated patients, phase II, those resistant to amethopterin. What is plotted on the *ordinate* is percent remissions, the remission rate. The criteria used here are those proposed by the Cancer Chemotherapy National Service Center. For children, the responses in phase II were not significantly different from the responses in phase I for either drug, though they were slightly better in phase II. This is balanced by the slightly greater occurrence of steroid usage in phase I in both drug-treatment programs. For adults, the remission rate is much lower with amethopterin and, for 6-MP, remission rates are not significantly different whether conditioned by amethopterin or not.



TEXT-FIGURE 2.—Effect of stage of disease and previous treatment on response to therapy. *Solid bar,* complete remission; *crosshatched bar,* partial remission; *open bar,* remission after group received steroids.

We can conclude from this preliminary analysis that resistance or refractoriness to a drug does not condition the response rate to the second agent, provided it is a different class of activity. More important is that the patients in phase II give us a good estimate of the activity of the drug. In other words, it was not essential that we use untreated patients to evaluate the effectiveness of either of these two agents.

We can look at this another way by examining survival at the end of the study. In text-figure 3 we have plotted the survival from the onset of the phase of therapy, that is, from the onset of phase I and from the onset of phase II treatment. For both children and adults, the survival from the onset of the study was qualitatively and quantitatively the same, whether the drug was used as initial treatment or after conditioning. Extrapolating from this experience, we can say that, if we had another agent, a third class of activity which was different from the first two agents that we are talking about, it would seem reasonable to study this agent in patients refractory to the other two agents. We should be able to get an estimate of its activity.



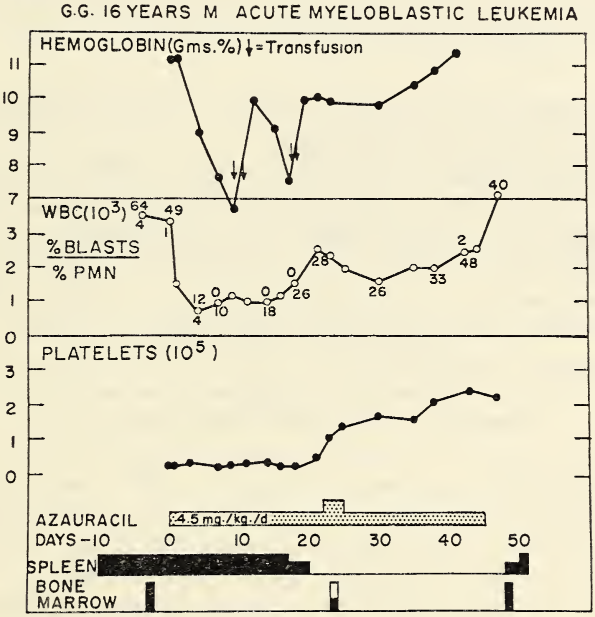
Text-figure 3.—Effect on survival of stage of disease—phase I (*solid* line) versus phase II (*broken* line)

A new agent that our group has studied in patients with advanced, refractory, acute leukemia is 6-azauracil, a pyrimidine antagonist which was suggested for clinical trial by Dr. Arnold Welch. What are the problems that arose when we studied this new agent? We had the animal data which told us that it was effective against certain transplanted animal tumors, and showed little toxicity. When azauracil was used clinically, we were surprised to find that the drug had a unique toxicity. The drug produced profound central nervous system depression in man at less than 10 percent of the dose that an animal could tolerate with ease.

This observation suggests another major problem in selecting drugs for trial: The animal screen not only sometimes fails us in predicting antitumor activity but can also fail us in predicting toxicity of the drug for man. Nonetheless we felt that, since the pyrimidine antagonist was a new area of activity, it was still an important drug to study, even though the dose that we could use was a small fraction of the dose required in the animal to produce an antitumor effect.

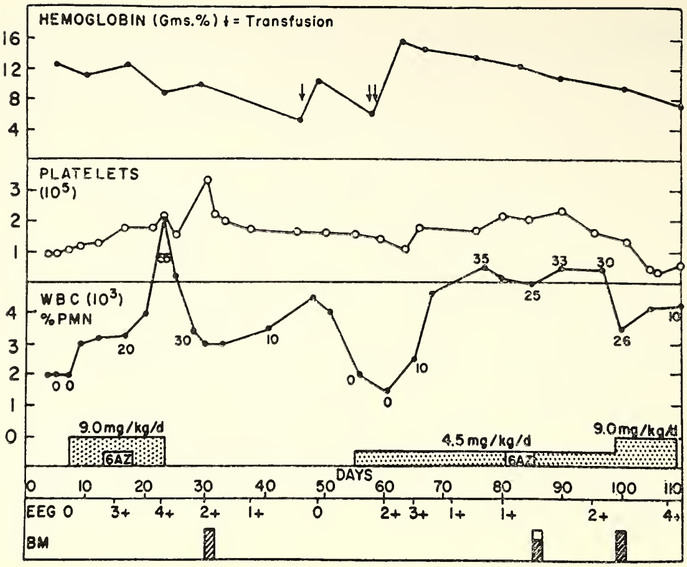
During the initial toxicity studies, we observed a number of interesting phenomenon that I would like to illustrate with the clinical courses of 2 patients who received 6-azauracil in the toxicity study.

First, a 16-year-old boy with acute myeloblastic leukemia, refractory to both 6-MP and amethopterin, at the onset of therapy had severe thrombocytopenia and 64 percent blasts with a white blood count of about 4,000 (text-fig. 4). He had a significant transfusion requirement. After the initiation of azauracil therapy the improvement was obvious. His marrow improved, the percent abnormal cells decreased, platelets rose, blasts disappeared from the circulating blood, and transfusion requirement disappeared. This response seemed directly related to the administration of 6-azauracil.



Text-figure 4.—Effect of 6-azauracil on 16-year-old male with acute myeloblastic leukemia. *Arrows* above hemoglobin line, transfusions required.

Second, a 5-year-old boy with acute lymphoblastic leukemia showed a rise in platelets and increase in neutrophils in the circulating blood (text-fig. 5). The drug was stopped because of neurotoxicity on day 23, after which there was a prompt recrudescence of disease: Blasts in the peripheral blood recurred, platelets fell somewhat, and transfusion requirement appeared. He was restarted on the drug and again showed



Text-figure 5.—Effect of 6-azauracil on 5-year-old boy with acute lymphoblastic leukemia

a rise in all formed elements in the blood and the reappearance of 20 percent normal elements in his marrow.

Because of these data in the toxicity study we felt that the question we wanted to answer was not whether this drug was as active as 6-MP or methotrexate and would produce 20 percent remissions, but whether 6-azauracil had any activity. Here was an area where the chemists could synthesize analogues of pyrimidines and give us other perhaps more active compounds, if we could show some activity. Certainly the detection of 20 percent remissions would not be too difficult, at least not as difficult as detecting any activity, perhaps 5 percent remissions. For this reason, we undertook a quantitative trial: Patients were assigned to two treatment groups, one receiving azauracil and the other receiving optimal medical care or a placebo (table 7). These were patients who were refractory to 6-MP and amethopterin, and all the children had received at least one course of steroids before this study. There was no difference between the 15 patients treated with 6-azauracil and the 13 getting placebo. More important, when the entire experience in the comparative study was compared to our experience in the pilot toxicity study, again they were qualitatively similar. As a result we had some estimate of how advanced acute leukemia, refractory to 6-MP, amethopterin and steroids, actually behaves.

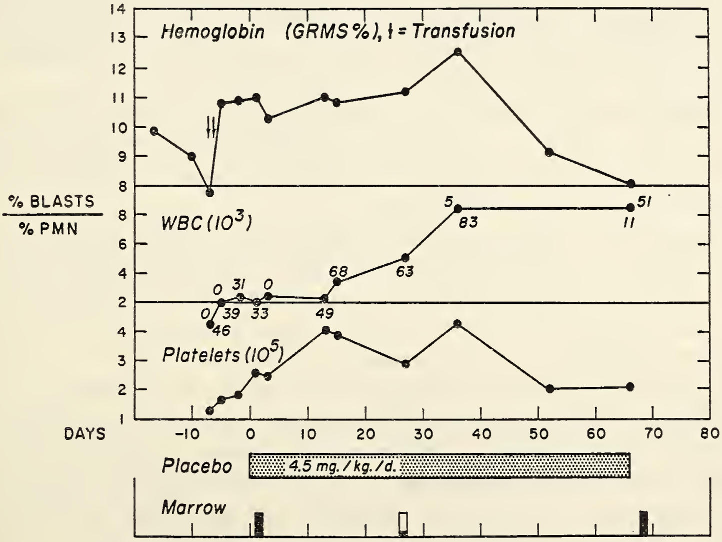
The course of the disease in patients does not inevitably and progressively decline, for there are interesting and dynamic changes occurring in this disease even in the advanced state. Rarely are there remissions by our criteria, but frequently there are significant falls in leukemia cells in the circulating blood, shrinkage of spleens, and rising platelets and these

TABLE 7.—Summary of hematological responses observed in comparative study of patients treated with 6-azauracil

Phase of study	Number of patients	Complete remission	Partial remission	Hematological improvement	Reduction of circulating leukemia cells	Total objective improvement (*percent*)
Comparative study:						
6-Azauracil	15	0	0	2	2	27
Control	13	0	1	1	2	31
Total	28	0	1	3	4	29
Noncontrol study:	23	1	1	3	2	30
Total	51	1	2	6	6	30

changes can occur spontaneously. The over-all frequency of some beneficial change occurring in the 5 patients that we studied was 30 percent, but I wish to emphasize that all these changes were minor in importance. Many of them were decreases in circulating leukemia cells in the blood. Remissions, when they occurred, were transient.

Text-figure 6 shows the effect of placebo administration on a 34-year-old woman with acute granulocytic leukemia, refractory to 6-MP and amethopterin. The patient showed a rise in platelets, disappearance of transfusion requirement, and reversion of marrow to less than 20 percent leukemia cells. The remission was maintained for less than 4 weeks and was qualitatively not important in the course of her disease. This



TEXT-FIGURE 6.—Effect of placebo administration on 34-year-old female patient with acute granulocytic leukemia, refractory to 6-MP and amethopterin.

is one of the better improvements. Others were even more transient, representing only rises in platelets, etc.

With this experience behind us we are prepared to continue to study new agents in this class of patients, keeping in mind that the detection of any activity represents a much greater investment than the detection of just activity of the order of 6-MP or amethopterin. This is a problem that we are facing with the study of Cytoxan. Dr. Sutow has already discussed his favorable experience. In some 90 patients with acute leukemia, we experienced a number of interesting changes. Several patients have fulfilled criteria for remission, but is this a drug of sufficient interest to us to invest in a comparative study so that we get quantitative data to understand its relative efficacy in relation to other alkylating agents and to the antimetabolites we now have?

In closing, I would say that although 6-azauracil was inactive in the form it was used, the riboside of 6-azauracil, 6-azauradine, is a compound now available, which we have given in doses up to 200 mg. per kg. without producing central nervous system toxicity. So we can reopen this area of the pyrimidine antagonists and examine this compound for antileukemic activity.

Dr. Whittington: Those of us from the 11 Veterans Administration hospitals participating in the acute leukemia study listened sympathetically to Dr. Gehan's paper. Four of the factors which he weighed most heavily in favoring the probability of remission were: age—our youngest patient was 21; type of leukemia (best is lymphoblastic)—90 percent of our patients were not lymphoblastic; sex (here again the advantage goes to the female)—our patients were all male; finally admission white blood count (WBC) (best to be below 10,000)—two thirds of our patients were above this level.

If one were "out" with 3 or 4 strikes in this ball game, we would have never come up to bat. Under the present rules, however, we have been in the league 3½ years and have stayed in the same relative position as your Washington baseball team.

Our study, to which 102 adult white males were admitted, compared the efficacy of two antipurines, 6-mercaptopurine and 6-chloropurine (CP). Our results, similar to those found at Memorial Hospital, showed that these drugs were almost exactly equally effective or, if you prefer, equally ineffective. A few general comments might be appropriate: Ninety-five percent of our patients were white, as compared with 5 percent Negro. This compares with about 25 percent over-all Negro population in our hospitals. However, the economic stress of the disease itself may affect these figures, so that this may not represent a true difference in racial incidence. Our patients' age range was from 21 to 79 years. Forty-five percent were over 60, and this bears out the fact that acute leukemia is primarily a disease of the extremes of age, the very young and the very old. The admission WBC was below 10,000 in 30 percent of our patients, while 25 percent were between 10,000 and 30,000 and 45 percent were above 30,000.

The survival data were extremely discouraging. Of 96 patients in whom the study was completed, the mean survival from the onset of symptoms was 22 weeks and from cellular diagnosis, 12 weeks. Fifty-six, or slightly more than one half of our patients, survived 4 weeks or more after admission to study. This group was considered to have had an adequate therapeutic trial of the antimetabolite. The mean survival of this group, *from admission to study,* was 15 weeks. We have employed a point score system for the last 2 years, which has been helpful as a guide line. We credit a patient with 8 points for each week of complete remission, 4 points for each week of partial remission, and 1 point for each week of clinical remission during the study period. The end point, in this study, was death. While this did not add information, *per se,* it did facilitate statistical evaluation and appealed to the bridge-playing members of our group.

Comparing the mean point scores in the 6-MP and the 6-CP patients, we found that the 6-MP patients had a mean point score of 10.3 and the 6-CP patients a mean point score of 10.5. This, of course, is essentially identical. I might mention, too, that our over-all rate of hematological remissions, either partial or complete, as defined by the Cancer Chemotherapy National Service Center criteria, was only 10 percent.

Finally, I would like to refer briefly to our recent work with intravenous 6-mercaptopurine riboside. I mention this because we were not able to get much data on the use of any antipurine intravenously in the human. Our experience to date has been with patients with malignant diseases other than acute leukemia, and we have found that the dose required to produce leukopenia is between 2 and 3 mg. per kg. per day for 21 to 28 days. Another interesting observation is that we have tried this, using a dose of 15 mg. per kg. weekly and twice weekly, and have seen either no leukopenia or moderate leukopenia after 8 weeks or more. To date we have not seen any nonhematological toxicity or antitumor effect in any patients treated with 6-mercaptopurine riboside.

I agree wholeheartedly with Dr. Burchenal that we should have uniform criteria for evaluating acute leukemia. I feel strongly that we need two sets of criteria: one for acute leukemia in adults and a second for acute leukemia in children.

Dr. Louis: The Midwest cooperative group was organized in February, 1959, and is composed of 18 institutions in the Middle West. The first study, begun September 1st, is a correlative study with the leukemia A group. It consists of the double-blind evaluation of thioguanosine and thioguanine versus 6-mercaptopurine in adult patients. It is too early to present any results from this study. The criteria for evaluation response to therapy are identical to the criteria developed by the leukemia A group. A phase-2 study, comparing various dose levels of cyclophosphamide, is also currently in progress. Preliminary results are based mainly on the data reported by the Ohio State University members. Cytoxan seems to have a relatively high degree of activity in Hodgkin's disease. Dr. Korst, Veterans Administration Hospital, Ann Arbor, Michigan, has reported favorable results with large doses of Cytoxan. Doses were as high as 20 mg. per kg. per day for 7-day intervals.

Other phase-2 studies are also in progress to determine the dosage and effectiveness of Sarcolysin, 1,1′-bis(2,3-epoxypropyl)-4,4′-bipiperidine (Lilly Compound 28002), and 1,4-bis(2,3-epoxypropyl)-piperazine (Lilly Compound 2163).

Dr. Burchenal: Dr. Mila Pierce, will you comment on patients treated first with steroids and later with new compounds?

Dr. Pierce: I would like to say a word of caution about the use of critically ill children for testing new compounds. There is a temptation to use new drugs on all untreated patients, but if we realize that a good evaluation of a new compound can really be made only on a child who is brought to at least a moderate disease status, it will help us to be more cautious and make the best use of the drug. In our group we use patients who are treated with steroids first and then allowed to relapse to at least an A-3 status of marrow; then we can evaluate a new drug in these patients as well as we can in untreated patients. Steroids should not be used to bring the patient only to a moderate disease status, and then the compound used immediately. The patient should be allowed to relapse so that at least the marrow status can be evaluated. But it does seem reasonable that, if a child is critically ill, the supportive treatment by the use of blood in transfusions is going to interfere with the evaluation of the drug as seriously as steroid initially.

Dr. Burchenal: Is there cross-resistance of Cytoxan with the other effective antileukemic agents? I believe that the men who have used this have worked with patients who are resistant to the other agents. Is that not correct, Dr. Sutow and Dr. Freireich? Therefore, I would say that there is no cross-resistance between Cytoxan and other agents.

The question is asked: What is Cytoxan?

Dr. Freireich: Cytoxan is a cyclic phosphoramide which has the alkylating group on a bis carrier group. It is an alkylating agent, however, and in the animal screens it does have phenomenal activity against transplanted mouse leukemia.

Dr. Burchenal: It is a bis-β-chloroethyl compound just like HN2 but, instead of a methyl group, there is a cyclic propylene phosphoramide.

How does the panel feel about the statement that adrenocortical hormones may cause exacerbation of some types of acute leukemia?

Dr. Sutow: I believe there are statements in the literature that monocytic or monoblastic types of leukemia are in some cases exacerbated by the use of steroids. We have seen a number of children who did not respond to the initial steroid, and whose condition steadily declined until they died. To what extent we can attribute the mortality to steroids, we do not know. We are hoping our study at least will give us some concept of using steroids in children who, on a morphological basis, statistically do not have a chance of responding.

Dr. Burchenal: We have heard a good deal about how we should try the effect of different dose routes and schedules of administration in animal tumors. A very good illustration is the work of Dr. Freireich's group with dichloromethotrexate.

Dr. Freireich: Dichloromethotrexate is a dihalogenated methotrexate, which Dr. Goldin described in mouse Lymphoid leukemia L1210 as having a qualitatively and quantitatively different effect than amethopterin. Mice could be, by all criteria, "cured" of their disease, and this cure was not possible with any other treatment known at that time. When this agent was tried in man, its activity was not found to be strikingly different in the preliminary toxicity study, but, while these studies were going on, Dr. Goldin's group continued its work and discovered that this agent was only effective when given parenterally.

Dr. Burchenal: Has cyclophosphoramide ever been tried in massive single doses in acute leukemias on the basis of Goldin's work in L1210 leukemia, in which single doses seem more potent than repeated single doses, at least in early leukemia?

Dr. Sutow: We have had only one child who received large amounts within a short time—not in a single injection but in 3 daily doses of 20 mg. per kg., intravenously. The rather rapid drop in the white blood count caused us to discontinue this type of therapy. I do not know what a single large dose will do.

Dr. Freireich: The studies of the leukemia B group were almost directly designed to ascertain whether animal data could be used for man; *i.e.*, in the animal, a weekly dose was much more effective than a dose given daily. Our studies were set up to give the drug daily and weekly to alternate patients and to decide whether dosage schedule was as important in man as in the mouse. Our preliminary analysis indicates that the weekly dose is, in fact, somewhat superior to the daily dose, but we do not have the final analysis of this data.

Dr. Burchenal: We have barely touched the surface of criteria because Dr. Frei is going to discuss this later, but we might have a comment on it.

Dr. Louis: Developing objective criteria is one of the most important problems we have had in leukemia A and also in the Midwest groups. At present we are attempting to get uniform criteria in all the cooperative groups and I believe in a few months we will succeed. Probably of most importance was the establishment of what normal values are, and within leukemia A we have been able to do this on the basis of previous studies which we have performed.

Dr. Freireich: My own point of view is that criteria are unimportant. What we want to know is what drugs are active in leukemia. The tool in the language that we have to use is "criteria," so I think that, in this phase of our development, it is important only in that it is so automatic that we can use it effectively and not have to worry about it.

Dr. Burchenal: The question is asked: It is hard to understand the statement that survival is an inefficient criterion. Is it not more correct to state that there are more precise but less important accessory criteria? It seems to me that survival is certainly the most important criterion, but, again, one cannot wait in the evaluation of an agent for survival, particularly in some cases that go for very long periods.

In the past, acute leukemia was a relatively rare disease, and no single investigator had seen much of it. Now, in one group, 900 cases have been treated; in other groups, 200, 300, and so on. This is a tremendous opportunity for someone in these groups or from outside to study the natural history of the disease or to correlate the chemical findings with symptoms or with almost any other phase of the disease. Let us not let this great bulk of cases, with apparently rather excellent records, go to waste.

Investigations in Chronic Leukemia [1]

Chairman: DR. CHARLES M. HUGULEY, *Emory University, Atlanta, Georgia*

Members of Panel:

Dr. Dennis M. Donohue, ***King County Hospital, Seattle, Washington***
Dr. C. C. Shullenberger, ***M. D. Anderson Hospital, Houston, Texas***
Dr. Charles C. Sprague, ***Tulane University, New Orleans, Louisiana***

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[1] Presented at Conference on Experimental Clinical Cancer Chemotherapy, Washington, D.C., November 11, 1959.

Dr. Huguley: Regional groups have been engaged in the study of chronic myelocytic and chronic lymphocytic leukemias. I am in the Southeastern group. I wish to consider the usefulness of these particular illnesses for testing drugs. There are a variety of objective parameters which can be easily measured: changes in the blood counts, in the marrow, and size of the liver, the spleen and lymph nodes can all be measured. But just as with acute leukemia, it is sometimes difficult to apply criteria to the significance of changes in these parameters in terms of chemotherapeutic effectiveness.

Another feature of these illnesses is their relative stability. Over a period there may not be much change so that if a drug is used for 3 months in a short-term study, which is relatively ineffective, the patient probably will not become dangerously ill during this time. Furthermore, after a study has been completed on a short-term basis, the patient can be permitted to relapse moderately and, if willing, can be tested on a second and a third drug. We have tested as many as four drugs in some patients who are still quite enthusiastic about the procedure.

There is often not a great deal of difference between the status of the patient at the beginning of each of these successive studies, so that some definite value can be placed on the use of several drugs in a single patient. Another valuable characteristic of these diseases is that there is frequently a good response to a variety of drugs. Except for the hormones, almost all drugs which have had an effect on a solid tumor have also been demonstrated to have an effect in chronic myelocytic and often in chronic lymphocytic leukemia.

In leukemia, it is not as difficult for a drug to be effective as it frequently is in the treatment of solid tumors. Whether good effects from a drug in the treatment of chronic leukemia have any predictive value for its effect in other tumors is, of course, another question. However, these diseases do present a useful means of studying the chemotherapeutic effects of drugs. The very fact, however, that agents producing a good effect are available poses a problem which has already been mentioned in relation to acute leukemia. Since we have drugs which have a good effect, are we justified in putting patients on long-term studies with drugs of unproved value? We must not only look for new drugs which will have a good effect on these diseases but, since at the moment we lack completely effective new agents, we must also study the total long-term effects of agents currently considered to be good. Whether or not treatment prolongs life and whether it is better to treat continuously or intermittently are questions which have not been answered to the satisfaction of every one. Therefore, long-term studies for the duration of life are vital for our understanding of these diseases and their treatment.

Still, our chief problem is to find agents which are better than those we have now. Since survival studies take too long to give such an answer and are not justifiable with many new agents, we feel that short-term studies should be the major effort of groups interested in these diseases. Full chemotherapeutic effect can usually be reached and a judgment rendered in about 3 months. This may vary a little between the two diseases. Even when a long-term study is in process, it is possible to measure the short-term effect by the same criteria used in short-term studies and therefore obtain information regarding short-term effects of these drugs.

It is obviously possible that a drug, which might seem ineffectual in a short-term study of 3 months' duration, might actually be superior to other drugs in long-term effect. However, this possibility is not probable and does not seem to justify an insistence on survival studies as a criteria of relative effectiveness of drugs.

Dr. Donohue: The Western cancer group is investigating 2 drugs in the treatment of chronic lymphocytic leukemia, and 2 drugs in the treatment of chronic myelocytic leukemia. Our studies are designed to provide information on essentially 2 phases in the use of the compounds. The first phase is a short-term evaluation of the toxicologic and pharmacologic effect of the drugs, and encompasses roughly 4 months from the beginning of therapy. Continued therapy over the subsequent course of the disease will provide information on survival data in the same treated group of patients.

Under our protocol, patients are considered eligible for treatment who have white blood counts of 40,000 or greater. This is the primary criterion for inclusion in the study, with provisions for the elimination of patients who cannot be followed for a sufficient period of observation, and elimination of patients with complicating illness.

The data being presented represent an analysis done under the guidance of Dr. William Reynolds, our statistician, but the information has not been reviewed by other members in the group, and if there are inaccuracies these are my own responsibility rather than of the group as a whole.

In chronic myelocytic leukemia, we have compared demecolcine with methanesulfonic acid, tetramethylene ester (Myleran). The initial starting dose for both drugs was 0.066 mg. per kg. per day. It had been our hope that through increasing the dose in those patients who failed to respond to the initial dose we would obtain some type of dose-response relationship (table 1).

Table 1.—Chronic myelocytic leukemia: dose rate to control

Controlled (*percent*)	Mg. per kg. per day	
	Myleran	Demecolcine
60	0. 08	0. 08
80	0. 09	0. 12
90	0. 10	0. 17

However, the initial starting dose was close to that which would provide control in 60 percent of the patients receiving both Myleran and demecolcine. Table 2 shows that of all patients started on treatment, 89 and 76 percent in each instance were controlled. By control I mean the reduction of the white blood count from the initial starting level to a level of 24,000. In the initial treatment 2 early deaths occurred with Myleran, 1 patient was lost, 2 patients that received demecolcine died, and 4 were removed from the study before they had sufficient therapy to expect a response.

TABLE 2.—Chronic myelocytic leukemia

Drug	Total patients	Number controlled*	Controlled (*percent*)
Myleran†	25	22	89
Demecolcine‡	25	19	76

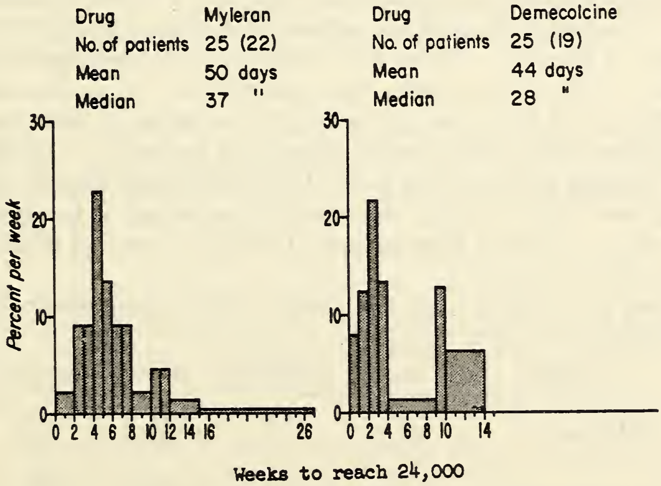
*White blood count<24,000.

† Two early deaths.

‡ Two deaths; 4 removed from the study.

In analyzing the data we have estimated the white blood count response time for each drug. It can be seen from text-figure 1 that the mean time required for the white blood count to fall to a level of 24,000 is not significantly different between Myleran and demecolcine. You will notice a biphasic hump in the distribution of patients treated with demecolcine. We think that this is due to the fact that investigators were hesitant to increase the dose when the patient did not initially respond. Therefore, the time was increased before the blood count fell to the control level.

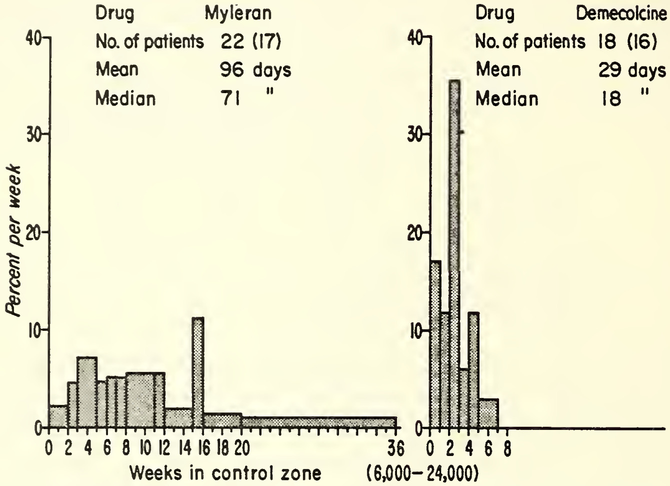
Between the time that the white count had reached 24,000 and had fallen to 12,000, the drug was stopped. We then observed the period that



TEXT-FIGURE 1.—Estimated white blood cell response times in chronic myelocytic leukemia.

the patient remained in remission, *i.e.*, the time before the white count had again risen to 24,000.

This period of remission presents a significant statistical difference, and the only one observed in these studies, in terms of the mean with which the patients were in the control zone: 96 days with Myleran as compared to 29 days with demecolcine (text-fig. 2).



TEXT-FIGURE 2.—Estimated length of white blood cell remissions in chronic myelocytic leukemia.

In addition to observations on the white blood count as an index of a positive pharmacologic effect of the drugs, we have estimated the hematocrit, the platelets, the size of the spleen, and the level of patient activity.

To consider that the hematocrit had been altered to be beneficial to the patient, we required (1) that the patient be anemic and (2) that the rise be at least 6 percent. There is no difference in the response by this parameter. With the platelets, we required that the count initially be below 100,000 and that it rise above 200,000, to allow for laboratory error. Only 1 patient in the entire group showed this response. The spleen was required initially to be at least 4 cm. below the costal margin, and to decrease by one half its initial measurement, in order to be considered a positive effect. Table 3 demonstrates that there were 14 of 20 patients

TABLE 3.—Chronic myelocytic leukemia: positive pharmacologic effect

Drug	>Hematocrit	>Platelets	<Spleen	>Activity
Myleran	$\frac{12}{21}$	$\frac{0}{1}$	$\frac{14}{20}$	$\frac{4}{8}$
Demecolcine	$\frac{11}{17}$	$\frac{0}{0}$	$\frac{13}{15}$	$\frac{2}{4}$

with large spleens, that had been treated with Myleran, who had a response, and 13 of 15 with demecolcine.

The grading of activity followed a scale ranging from 0 to 100 points that has been presented by Dr. David Karnofsky. It has been required by this scale that the patient have a score of 70 or less to be considered for an effect, and that the score rise by at least 20 points for the effect to be significant. There were not many patients in this group with a Karnofsky rating below 70 for the initial part of the study; thus no valid data for comparison of the drugs were provided.

Similar data have been accumulated on toxicity. For an effect to be called toxic, it was required that the hematocrit be normal at the start of therapy and fall by 6 percent. This did not occur during the period of observation with Myleran or demecolcine. The platelets, likewise, were required to be at least 200,000 and to have fallen to 100,000 or below, to be considered a toxic drug effect. This happened 4 times with Myleran and once with demecolcine. The difference is not statistically significant.

Leukopenia was considered as a toxic manifestation if the blood count fell to 3,000. This happened 2 times with demecolcine. Neither patient died as a result of leukopenia, but both were very ill. In addition, 1 patient on demecolcine was removed from the study because of nausea and vomiting. No other patients were removed from the study because of toxic effects, *e.g.*, nausea, vomiting, diarrhea, and skin rashes.

Table 4.—Chronic myelocytic leukemia: toxic effect

Drug	Hematocrit	Platelets	Leukopenia (<*3,000*)	Other
Myleran	$\frac{0}{21}$	$\frac{4}{20}$	$\frac{0}{21}$	$\frac{0}{21}$
Demecolcine	$\frac{0}{17}$	$\frac{1}{17}$	$\frac{2}{17}$	$\frac{1^*}{17}$

*Nausea and vomiting.

In chronic myelocytic leukemia, the only difference in the initial pharmacologic and toxicologic comparison of the two compounds has been the difference in duration of remission, considerably shorter with demecolcine than with Myleran.

The same type of data are presented for chronic lymphocytic leukemia, with the same criteria for positive pharmacologic and toxic effects. Again the initial doses were sufficient to control the majority of patients at the initial dose rate. Triethylenemelamine (TEM), prescribed at 0.07 mg. per kg. per week, controlled 88 percent of the patients, *i.e.*, they all responded at this or a lower dose. Chlorambucil at 1.40 mg. per kg. per week or less controlled 98 percent of the patients (tables 5 and 6).

The estimated length of white blood cell remission with chlorambucil and with TEM is not significantly different, a median of 81 and 93 days (text-fig. 3).

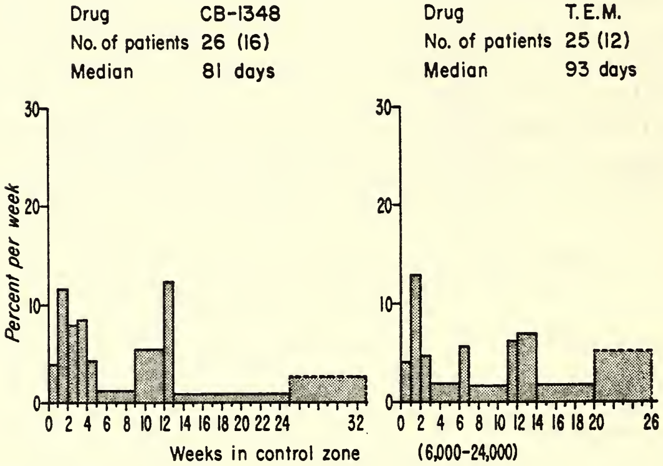
Table 5.—Chronic lymphocytic leukemia

Drug	Total patients	Number controlled*	Controlled (*percent*)
TEM †	30‡	24	83
CB 1348	31	27	87

*White blood count <24,000.
†Two toxicity, 2 steroids, and 2 no response.
‡Three no response, 1 lost.

Table 6.—Chronic lymphocytic leukemia: dose rate to control

Drug	Mg. per kg. per week	Controlled (*percent*)
TEM	0. 07	88
CB 1348	1. 40	98



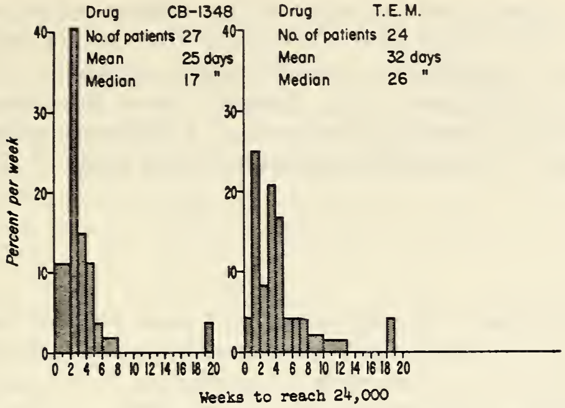
TEXT-FIGURE 3.—Estimated length of white blood cell remissions in chronic lymphocytic leukemia.

The white blood cell response time—the time required for the blood count to reach a level of 24,000—is comparable, 25 and 32 days. This difference cannot be considered significant (text-fig. 4).

The pharmacologic effect, with the use of the same criteria as outlined under chronic myelocytic leukemia, again shows no significant difference between the two compounds (table 7).

Table 7.—Chronic lymphocytic leukemia: positive pharmacologic effect

Drug	>Hematocrit	>Platelets	<Spleen	<Nodes	>Activity
TEM	$\frac{2}{9}$	$\frac{0}{2}$	$\frac{9}{16}$	$\frac{4}{6}$	$\frac{2}{4}$
CB 1348	$\frac{1}{10}$	$\frac{1}{6}$	$\frac{8}{10}$	$\frac{3}{5}$	$\frac{3}{6}$



TEXT-FIGURE 4.—White blood cell response times in chronic lymphocytic leukemia.

The failure to improve the hematocrit is observed with both TEM and chlorambucil: in only 2 of 9 instances with TEM and 1 of 10 with chlorambucil. The hematocrit rose when it was initially low by 6 percentage points. The response was excellent in the reduction of spleen sizes, and also in the reduction of node size. The toxic effects in chronic lymphocytic leukemia are shown in table 8.

Table 8.—Chronic lymphocytic leukemia: toxic effect

Drug	Hematocrit	Platelets	Leukopenia (<*3,000*)	Other
TEM	$\frac{4}{16}$	$\frac{6}{23}$	$\frac{0}{24}$	0
CB 1348	$\frac{3}{12}$	$\frac{4}{16}$	$\frac{1}{22}$	1*

*Exfoliative dermatitis.

Four of 16, and 3 of 12 patients on the respective drugs had a significant fall in hematocrit while being treated. The effect on platelets and the development of leukopenia in 1 patient treated with chlorambucil is noted. In addition, 1 patient was removed from the study because of exfoliated dermatitis; none of these observances are statistically significant. The drugs appear comparable in this short-term phase of the study, insofar as toxicity is concerned.

In summary, this initial analysis of data has described the toxicologic and pharmacologic effects of two drugs in the treatment of chronic myelocytic and chronic lymphocytic leukemia. The period of observation included the initial response phase to a level of 24,000 total white blood count; then the duration of remission during which the patient's white blood count remained at or below 24,000 without subsequent therapy. The initial observations have shown only one statistically significant difference, *i.e.*, the duration of remission with colcemide, or demecolcine, as compared to Myleran. This obviously raises the question of what the difference is in these two compounds. A difference which might well be biologically, or chemically, significant in the mode of action of these drugs.

Dr. Shullenberger: Dr. Sprague and I have been designated representatives of the Southwest cancer chemotherapy study group, to present the results obtained by the group in two studies: (1) on the comparative effects of Myleran and 6-mercaptopurine (6-MP) in the management of chronic myelocytic leukemia, and (2) on the comparative effects of chlorambucil and P^{32} in the management of chronic lymphocytic leukemia. Briefly, the group is composed of representatives of 11 institutions in Arkansas, Oklahoma, Texas, and Louisiana. The group is indebted to its chairman, Dr. Grant Taylor, and to its headquarters' staff, headed by Dr. Roy C. Heflebower, for the continuity of its effort. It is also greatly indebted to its evaluation committee, Drs. W. C. Levin, James Hammersten, Phil Davis, and Miss Eleanor MacDonald, the biostatistician and chaired by Dr. S. W. Ross.

Because the group's approach to evaluation of results is basically the same in each of these two studies, I will outline briefly the method of evaluation for both; then I will discuss our results in chronic myelocytic leukemia and Dr. Sprague will present the chronic lymphocytic leukemia results.

In the chronic myelocytic leukemia study, the comparison was made between 6-MP and Myleran. Why these two drugs? This study was undertaken just over 2 years ago, and these were the outstanding drugs available for comparison at the time. The group was in the formative stage and this type of study appeared best suited to start a large cooperative effort. In addition, it appeared indicated to test Myleran, already considered at that time the standard drug for therapy of this disease, and functionally an alkylating agent, against a drug of an antimetabolite nature which had demonstrated definite activity in control of the disease. Randomization in the selection of therapy results in following pairs of patients, 1 of the pair receiving Myleran and the other, 6-MP. Briefly, with Myleran the initial dose was 6 mg. daily, and this might be increased to 8 or 10 mg., depending upon the patient's sensitivity or resistance to the drug. With 6-MP the initial dose was the standard 2.5 mg. per kg. per day with provision being made for increase to 3.75 and then to 5 mg. per kg., over a period of weeks, if this became necessary. With both drugs provision was made for adjusting the daily dose, dependent on response, with the aim at a maintenance dose which would keep the leukocyte count between 5,000 and 15,000, and for switching to the other drug if the one would not control the clinical and hematologic manifestations of the disease.

A total of 60 patients has been followed in the chronic myelocytic leukemia study according to this protocol. In chronic lymphocytic leukemia, the basic procedure results in pairing of the patients receiving chlorambucil and P^{32}; a total of about 120 patients has been followed. Dr. Sprague will outline the dosage schedules used in this latter group.

In both studies the following categories of response were utilized: (1) complete remission; (2) partial remission, which was subdivided into improved, unchanged, and worse; (3) no remission, also subdivided into improved, unchanged, and worse; and (4) relapse. In the evaluation of response to therapy, two difficulties were encountered: (1) The category "partial remission" was so inclusive that determination of significant improvement or deterioration was often hardly possible; (2) an original plan to separate the patients into previously treated and untreated groups caused an arbitrary distinction which seemed unrealistic, since some previously treated patients were yet in the early phase of their disease while others, though previously untreated, were in advanced stages. Therefore, the biostatistician and the evaluating committee evolved a system of scoring in which each parameter was given a numerical equivalent that was weighted so as to increase in accordance with the severity of the symptom or finding.

Table 9 shows the method of scoring for the evaluation of these patients, which serves as the basis of the reports made by Dr. Sprague and myself. Six parameters were followed and they were assigned varying scores according to the judgment of the committee with regard to the relative severity of the particular symptom or finding. Note that considerable weight is given to the activity status, the simple basis being

TABLE 9.—Method of scoring for evaluation*

Hemoglobin (*gm. %*)	Score	Liver (*cm.*)†	Score
12 plus	0	0	0
10–12	1	<7	1
7–10	3	7–14	3
<7	6	14 plus	6
White blood count (×1,000)		Spleen (cm.)†	
3–15	0	0	0
15–50	1	1–10	1
50–300	2	10–19	2
300 plus	3	20 plus	3
<3	3	Splenectomy	1
Platelets (×1,000)		Activity	
125–1000	0	Bedridden	12
1000–3000	1	Hospital, ambulatory	6
3000 plus	3	Home, ambulatory	3
90–125	1	Unrestricted	0
<60	6		

* Total score of 0 to 9.9 corresponds to "early disease"; total score of 10 plus corresponds to "late disease."

† Below the coastal margin in midclavicular line.

that if the patient's leukemia is so bad that he is confined to bed this parameter must, of necessity, be given heavy weight. These 6 parameters formed the basis for our reports both in the chronic myelocytic leukemia patients and in the chronic lymphocytic group. One may say that these are certainly arbitrary values, and no argument will be raised to such a criticism. It can only be said that in the opinion of the committee, which has followed these cases very closely, the scores of these patients in these various parameters have reflected closely their clinical status over the course of the study. With this method of evaluation and the division of the cases, 2 groups are made available for study: (1) those whose initial score, on entering the study, is 0 to 9.9; (2) those whose initial score is 10 or more. There appears to be a remarkable correlation between duration of disease and initial score, those with 0 to 9.9 being relatively early in their course, those with 10 or more being relatively late. Each of the 6 parameters was tested at monthly intervals to see if there was a difference in response by those treated with one drug as against those treated with the other.

For the evaluation of the results of therapy in the patients in the chronic myelocytic leukemia study, the analysis in table 10 is prepared on the basis of total patient days in each of the categories and summarizes the status of the patients in the Myleran-treated group and in the 6-MP-treated group at the end of 6 months on this study. The significant findings here are: (1) The 6-MP-treated group shows a significantly higher number of patient days in complete remission; (2) the Myleran-treated group shows a significantly higher number of days in the "partial remission improved" category; and (3) there is no difference in the "relapse" category.

TABLE 10.—Chronic myelocytic leukemia: summary of status of patients at 6 months, by treatment

		Myleran		6-MP	
		(days)	*(percent)*	*(days)*	*(percent)*
Complete remission		359	12. 5	566	19. 8
Partial remission	Improved	1, 120	39. 2	802	28. 1
	Unchanged	356	12. 5	442	15. 5
	Worsened	132	4. 6	255	8. 9
No remission	Improved	240	8. 4	69	2. 4
	Unchanged	134	4. 7	136	4. 8
	Worsened	88	3. 1	160	5. 6
Relapse		427	15. 0	426	14. 9
			100. 0		100. 0

Table 11 shows the summary of patient days in the various categories at the end of 12 months. Here, the situation is essentially reversed, with the Myleran-treated group showing a significantly higher number of days in complete remission and a significantly lower number in relapse. There are no marked differences in the other categories.

Table 11.—Chronic myelocytic leukemia: summary of status of patients at 12 months, by treatment

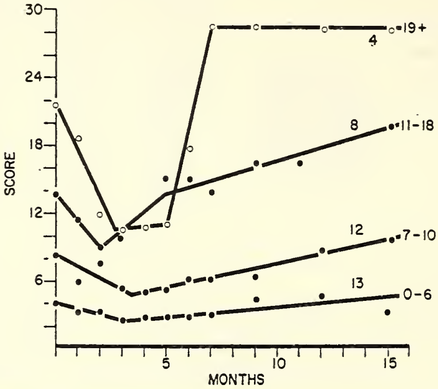
		Myleran		6-MP	
		(days)	*(percent)*	*(days)*	*(percent)*
Complete remission		911	20. 9	440	10. 1
Partial remission	Improved	1, 029	23. 6	1090	25. 0
	Unchanged	789	18. 1	749	17. 1
	Worsened	373	8. 5	440	10. 1
No remission	Improved	251	5. 7	75	1. 7
	Unchanged	113	2. 6	128	2. 9
	Worsened	119	2. 7	195	4. 5
Relapse		783	17. 9	1251	28. 6
			100. 0		100. 0

When one applies the tests of significance to the effects of the drugs on the 6 individual parameters, the summary shown in table 12 is obtained. The "early" group here is composed of patients with an initial score of 0 to 9.9, the "late" group of those with an initial score of 10 or more. Myleran appears to control hepatomegaly and leukocytosis better than 6-MP in the earlier stages of therapy in the early group; 6-MP appears to have a better effect in maintaining a normal platelet count during part of this period. After 9 months of follow up, there are no significant differences in the effects of the drugs on the various parameters in this group. In the late group, there are no significant differences in the drug effects at any time during the 15-month period of follow up.

Table 12.—Chronic myelocytic leukemia: significant difference according to treatment in 6 parameters

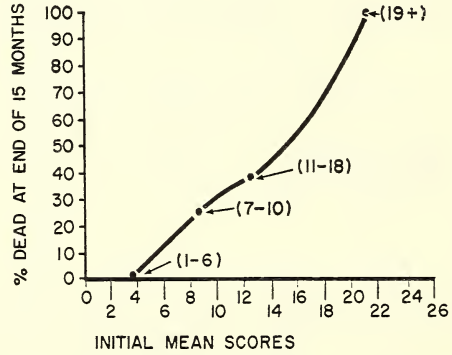
	Early		Late	
Months	Myleran	6-MP	Myleran	6-MP
3	Hepatomegaly	—	—	—
6	White blood count	Platelet	—	—
9	White blood count	—	—	—
12	—	—	—	—
15	—	—	—	—

Text-figure 5 shows graphically the course of groups of patients categorized on the basis of their initial score, regardless of the drug used. In each group, during the first 4 to 5 months of therapy, there is an improvement in the mean score, considerably more marked in the groups with initial high scores. After this, in all groups, there is a progressive rise in the mean score during the remainder of the 15-month follow-up period; the most striking line in the graph is, of course, that of the group with an initial score of 19 plus, which shows a marked improvement in the mean score during the first few months of therapy, followed by marked deterioration and rapid return to the high-score status. The small size of this latter group, as compared to the others, however, suggests caution in drawing a general conclusion. A generalization which does appear valid is that over this period of follow up, regardless of the drug employed, there is a very marked tendency for the low-score groups to remain in that category, and for the high-score groups to remain so.



Text-figure 5.—Mean myelocytic leukemia scores by groups. Numbers *above,* or *below,* lines indicate number of patients in the group. Figures at *ends* of lines indicate scores by groups.

In text-figure 6 an analysis of the death rate by groups simply shows that, at the end of the 15-month follow-up period, the group with the lowest initial score has sustained no deaths, while in the initial high-score group the mortality is practically 100 percent; the gradient, representing the increasing mortality by group scores, is steep.



Text-figure 6.—Myelocytic leukemia death rate by groups according to initial score. Numbers at *points* on the line indicate scores by groups.

To summarize briefly, it appears that Myleran shows some superiority when measured by the criteria of day-to-day management of this disease, during the earlier phase of therapy. The following conclusions also appear warranted on the basis of the results of the study to date: (1) Regardless of which of the drugs is used, those patients in whom therapy is begun in the early stage of their disease show the least tendency to progression of disease during the first 15 to 18 months of treatment; (2) it appears doubtful that either drug significantly affects the course of the disease in those patients in whom the disease is relatively advanced at the time therapy is initiated; (3) one must still seriously consider the proposition that prolongation of survival is the ultimate test of the true efficacy of therapy in this disease. Yet, this study and other similar ones will furnish a solid background against which the further search for more effective agents in the treatment of chronic myelocytic leukemia may more rationally be made.

Dr. Sprague: Dr. Shullenberger has presented the method of statistical analysis and I can dispense with that portion of the chronic lymphocytic leukemia study, inasmuch as the chronic myelocytic and chronic lymphocytic leukemia patients were analyzed in essentially the same manner. What we have learned is not so much the differences in effectiveness of drugs, as what we have learned of the natural course of chronic lymphocytic leukemia and the methods of evaluating patient response. This knowledge should prove valuable in the planning of future studies.

Only patients who were anemic or who had symptoms attributable to their leukemia were admitted to the study.

The two agents evaluated were radioactive phosphorus and chlorambucil (Leukeran, CB 1348). The radioactive phosphorus was administered according to the method as outlined by Dr. Osgood. Regularly titrated doses of P^{32} were administered intravenously in an attempt to maintain optimum control of the leukemia. If the patients were not in a partial remission at the end of 18 weeks of therapy they were considered treatment failures.

With chlorambucil, the initial dose was 0.2 mg. per kg. of body weight. If after 6 weeks of therapy the initial white blood count had not fallen to below 50 percent of the initial value, the dose was increased by 0.1 mg. per kg. If after an additional 6 weeks of therapy, the white blood cell count was not below 25,000 per mm.^{3}, the dose was increased to a total of 0.4 mg. per kg., and after the 18th week of therapy if the patients still failed to fulfill the criteria for partial remission they were considered treatment failures.

The status of patients at the end of 6 months of therapy is summarized in table 13. The significant differences at this interval are indicated in table 14.

At 6 months, radioactive phosphorus was slightly superior to chlorambucil.

A similar analysis of the status of patients at the completion of 12 months of study is presented in tables 15 and 16. Again, at this interval radioactive phosphorus seemed to be slightly superior to chlorambucil.

In the early phases of this study it became apparent that this method of evaluation was not particularly satisfactory and that the separation of patients into treated and untreated categories bore little relationship

TABLE 13.—Chronic lymphocytic leukemia: summary of status of patients at 6 months, by treatment

	CB 1348		P^{32}	
	(days)	*(percent)*	*(days)*	*(percent)*
Complete remission	84	1. 3	14	0. 2
Partial remission improved	1, 984	30. 3	2, 631	40. 2
Partial remission unchanged	1, 949	29. 7	1, 472	22. 4
Partial remission worsened	490	7. 5	458	7. 0
No remission improved	117	1. 8	172	2. 6
No remission unchanged	473	7. 2	595	9. 1
No remission worsened	147	2. 2	262	4. 0
Relapse	1, 308	20. 0	948	14. 5
		100. 0		100. 0

TABLE 14.—Chronic lymphocytic leukemia (CB 1348–P^{32}): significant difference at 6 months according to summary of status

Status	Result of treatment
Partial remission improved	Higher on P^{32}
Partial remission unchanged	Higher on CB 1348
Relapse	Lower on P^{32}
No other differences	

TABLE 15.—Chronic lymphocytic leukemia: summary of status of patients at 12 months, by treatment

	CB 1348		P^{32}	
Status*	*(days)*	*(percent)*	*(days)*	*(percent)*
Complete remission	506	5. 4	52	0. 6
Partial remission improved	2, 341	24. 9	3, 242	34. 5
Partial remission unchanged	2, 204	23. 4	2, 360	25. 1
Partial remission worsened	525	5. 6	691	7. 3
No remission improved	97	1. 0	103	1. 1
No remission unchanged	562	6. 0	580	6. 2
No remission worsened	149	1. 6	174	1. 8
Relapse	3, 024	32. 1	2, 206	23. 4
		100. 0		100. 0

*The days of each type of remission will sometimes be less at 12 than at 6 months because there are fewer pairs at 12 than at 6 months.

TABLE 16.—Chronic lymphocytic leukemia (CB 1348–P^{32}): significant difference at 12 months according to summary of status

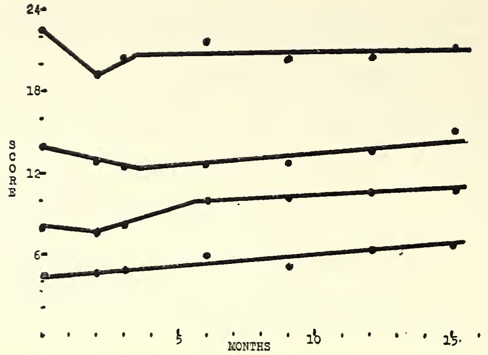
Status	Result of treatment
Partial remission improved	Higher on P^{32}
Relapse	Lower on P^{32}
No other differences	

to the severity of their disease at the time they were admitted to the study. As a result, a scoring system similar to that presented by Dr. Shullenberger was used for the purpose of evaluating patients as they came into the study as well as for evaluating their response to therapy. The patients who had minimal manifestations of their leukemia (low score) were termed early cases and those with more severe involvement (high score) termed late. The response of patients in the early and late categories in terms of the various parameters measured at 3-month intervals is summarized in table 17. There is the suggestion here that chlorambucil seemed to have a more favorable effect on the peripheral blood values than did P^{32}, whereas P^{32} may have had more effect on the lymphadenopathy and splenomegaly.

TABLE 17.—Chronic lymphocytic leukemia: significant difference according to treatment in six parameters

	Early		Late	
Months	CB 1348	P^{32}	CB 1348	P^{32}
3	White blood count	Platelet	—	—
6	White blood count	—	Hepatomegaly	Activity status
9	White blood count	Lymphadenopathy	White blood count	—
12	Hemoglobin	Splenomegaly	—	—
15	Hemoglobin	Lymphadenopathy	—	—

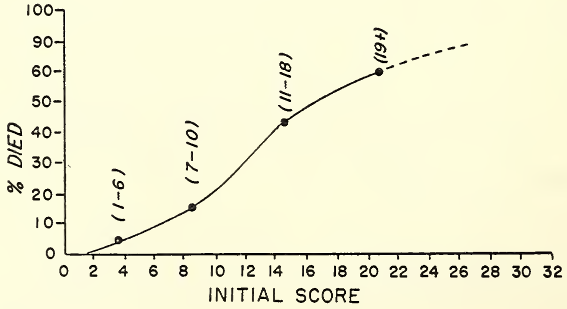
One of the most interesting results of the scoring method of analysis was to note the course of patients depending upon their score (severity of disease) at the time they were admitted to the study, irrespective of the type of therapy. The patients were arbitrarily divided into 4 categories, those with initial scores of 0 to 6, 7 to 10, 11 to 18, and 19 and above. There were 23 patients in each of the first 3 categories and 17 in the fourth. The scores of all the patients in each category were averaged at 3-month intervals and plotted to see how the course of the disease might have been altered. A favorable response to therapy would be reflected by a decrease in score, hence a downward slope in the curve. The average scores are plotted for the 4 categories of patients in text-figure 7. Interestingly, there was no descent from one category to a lower, more favorable one in any of the 4 categories. This differs from what was observed in chronic granulocytic leukemia as demonstrated in Dr. Shullenberger's presentation. There were individual patients, of course, whose scores would fluctuate sufficiently that they did not remain in the same category throughout the study, but when the average or the mean score of the group was plotted there was no overlap. This suggested, but was in no sense proof, that the therapy employed had altered the course of the patient's disease very little, though significant differences in individual parameters were noted.



Text-figure 7.—Mean lymphocytic leukemia scores by groups.

Additional evidence to suggest that the scoring method had validity in terms of predicting the future course of the disease was the mortality rate observed in the various categories (text-fig. 8). In the 0 to 6 score category there was only 1 death (4% mortality), whereas in the group of patients with an initial score greater than 18 there were 65 percent dead at the end of 18 months. If these impressions can be confirmed by data from a much larger sample, it would seem that this knowledge of the course of chronic lymphocytic leukemia patients treated by radioactive phosphorus or chlorambucil might well serve as a reference standard to judge the effectiveness of new agents. It is quite possible that different types of analysis could be carried out simultaneously in which patients were randomized in each of the 4 categories, according to their score upon entry into the study. In those with initial scores in excess of 18, where an 18-month mortality rate of approximately 65 percent may be anticipated, it may be feasible to use survival as the end point in evaluating 2 agents. At the same time data from patients in this category could be considered with data from categories 2 and 3, if the same agents and treatment schedules had been employed in all 3 groups.

In summary, it is fair to say that we have been able to demonstrate very little difference in the effectiveness of radioactive phosphorus and chlorambucil in the treatment of chronic lymphocytic leukemia; radio-



Text-figure 8.—Lymphocytic death rate by groups.

active phosphorus may be slightly superior in over-all effectiveness. Perhaps the most important information to come from the study concerns the scoring method of evaluation. If additional data bear out the impressions to date, then this method of evaluation should prove valuable in planning future studies.

Dr. Huguley: I would like to emphasize that the studies so far reported have been comparisons of drugs which are thought to be effective, and the failure to demonstrate definite differences consistently on a short-term study does not deny the usefulness of short-term studies in these diseases. It simply means that when drugs are similar, survival data are necessary for defining differences. In the interest of time, I am not going to dwell on a long-term study that the Southeastern group is doing. We have concentrated on short-term studies. I would like to say, though, that we do have a long-term study in chronic lymphocytic leukemia and we have felt that it was desirable to set it up not only with different drugs but also with different treatment regimens. Rather than treat all patients continuously, we have set up a continuous "maintained" therapy schedule and, in addition, an "intermittent" schedule in which patients are treated only for certain specific disabilities, treatment being stopped as soon as this limited objective is obtained, with retreatment again when relapse occurs. So far, we have not enough patients enrolled to present any definite data on this study.

We have set up several short-term studies with evaluation after 3 months of treatment. In chronic lymphocytic leukemia, our first study was the relative effectiveness of chlorambucil and Myleran. When we started this, it was a general impression that chlorambucil was good in chronic lymphocytic leukemia and Myleran was not. The initial doses were 6 mg. per day of Myleran versus 12 mg. per day of chlorambucil. The dose was subsequently varied at stated intervals by increments of one third so that Myleran was varied by 2 mg. per day and chlorambucil by 4 mg. per day. This permitted the use of a double-blind technique. We attempted to bring the total white count to as nearly normal as possible without serious depression of granulocytes, hemoglobin, or platelets, and we analyzed the status of the patient at the end of 3 months. We were looking for the ability to produce a change and maintain it for at least that long.

The results are shown in table 18. The criterion of "excellent" was an almost completely normal hematologic picture with all other evidences of disease being absent, whereas "good," "fair," and "questionable" changes were not entirely absolute but were defined in terms of improvement over the previous status, good being at least 50 percent improvement in all measured parameters, fair, at least 25 percent, and questionable, the situation in which we had definite improvement in some parameters but not in all.

TABLE 18.—Summary of therapeutic response of patients with chronic lymphocytic leukemia to chlorambucil and Myleran*

Response	Myleran	Chlorambucil
Excellent	0	3 (13%)
Good	0	12 (52%)
Fair	3 (18%)	2 (9%)
Questionable	9 (53%)	6 (26%)
None	5 (29%)	0
Total number of patients	17	23

*R. W. Rundles *et al.*: Comparison of chlorambucil and Myleran in chronic lymphocytic and granulocytic leukemia. Am. J. Med. 27: 424–432, 1959.

With chlorambucil we obtained, in 3 months, results that were defined as good or excellent in 65 percent of these patients. With Myleran none of the patients fell into those categories. We believe that this is highly significant and we can say, with 95 percent confidence, that under the terms of the study 54 to 88 percent of patients would have a fair or better response to chlorambucil, whereas only 5 to 44 percent could be expected to respond as well to Myleran.

The average hematologic data are even more striking. With Myleran we were unsuccessful in achieving our objective of a normal white blood count. With chlorambucil we were able to bring it down on average to 10,000. However, in spite of the failure to accomplish this much change in the white count, the dose of Myleran given produced a lower hemoglobin, a highly significant drop in the platelets, and definitely fewer granulocytes. The differences in production of these toxic effects by Myleran as compared to chlorambucil is highly significant in each of these categories.

While the results with chlorambucil are relatively good, we need a much more effective drug. We feel that if a drug is really good there is plenty of room at the top to demonstrate superiority to chlorambucil in a short-term study.

In chronic myelocytic leukemia, we did an identical study. We used the same dosage, varied in the same way, of 12 mg. of chlorambucil per day and 6 mg. of Myleran per day initially, varied by increments or decrements of one-third of the initial dose. The duration of the study was 3 months and the criteria of response the same as for the previous study.

The response here was not as striking but was still definite. All 21 patients treated with Myleran fell into the good or excellent categories, whereas with chlorambucil only 58 percent fell into that category (table 19).

The average hematologic data demonstrate better the difficulty in controlling a patient with chlorambucil as compared to Myleran. Myleran is pretty smooth, but chlorambucil requires seeing the patient frequently and varying the dose often. Even so, it was hard for us to bring the blood count down and keep it there because it is much more labile with chlorambucil than with Myleram. We accomplished our objective of a white cell count less than 15,000 with Myleran. We did not quite accomplish it with chlorambucil.

TABLE 19.—Summary of therapeutic response of patients with chronic granulocytic leukemia to chlorambucil and Myleran*

Response	Myleran	Chlorambucil
Excellent	8 (38%)	2 (9.6%)
Good	13 (62%)	10 (48%)
Fair	0	8 (38%)
Questionable	0	1 (4.8%)
Total number of patients	21	21

*R. W. Rundles *et al.*: Comparison of chlorambucil and Myleran in chronic lymphocytic and granulocytic leukemia. Am. J. Med. 27: 424–432, 1959.

With this limitation, we had somewhat better improvement in hemoglobin with Myleran than with chlorambucil. We brought the platelet count, on average, to normal with Myleran and not quite so with chlorambucil, and the effect on the total granulocytes was in line with the effect on the total white count. It would be difficult for a new drug used in chronic myelocytic leukemia to demonstrate superiority to Myleran in a short-term study, but it is obviously possible to show that a drug such as chlorambucil is inferior.

The statistical analysis of the results shown in table 19 is that, with 95 percent confidence, we can say that, with Myleran, 87 to 100 percent of the patients will have a good or an excellent response, whereas only 34 to 79 percent will do as well with chlorambucil.

We have also compared Myleran and 6-MP in a short-term study of chronic myelocytic leukemia. The dose is similar to that of the Southwestern group except that we were using 3 mg. per kg. per day of 6-MP instead of 2.5. Myleran was used in the same way as on the study reported. Because of our backlog of experience with Myleran, we randomized 2 patients on 6-MP to 1 on Myleran and this is our general procedure in testing new drugs. The dose was varied in the same way by thirds, the duration of study was 3 months, and the criteria of response the same. The study is incomplete. Table 20 represents the analysis of only 25 patients.

TABLE 20.—Summary of therapeutic response of patients with chronic myelocytic leukemia to Myleran and 6-MP*

Response	Myleran			6-MP
	This study	Past study	Total	
Excellent	3	8	11	1
Good	2	15	17	4
Fair	3		3	5
Questionable				7
None				
Total	8	23	31	17

*Incomplete study.

Myleran has not done quite as well in this study as on the past study. The total of the two studies is shown. Responses of two thirds of the patients were good or excellent, whereas with 6-MP only one third rated this classification. 6-MP has a fast effect, particularly on the platelets, and a fast rebound, and in our experience it was very difficult to keep the patient on a steady level. This study, as yet, is incomplete.

There are several obvious problems. One is the matter of comparative dosage. In solid tumors it is frequently possible to put a patient on a steady dosage schedule and keep him there for the duration of the study. This is not possible with chronic leukemia. The marrow is more sensitive to toxic effects and the response is more variable so that one starts with a standard dose and must be prepared to increase or decrease it in order to get a desired effect. Therefore, the criteria of adequacy of dose in each of these studies has been to rely arbitrarily on the production of a normal white blood count (below 15,000). The difference in the chemotherapeutic effect of two drugs would then be the relative effect on the parameters of disease activity and the relative toxic effect on marrow activity when a comparable reduction in the total leukocyte count has been achieved.

A second problem, which seems universal, is the establishment of criteria of therapeutic effect. The measurement of a variety of parameters is not difficult but evaluation of the significance of these changes is often highly subjective and there is no agreement among these groups as to the best way of evaluating results. Perhaps a weighing system such as used by the Southwest group will prove to be the best. It does seem, however, that the case data available in the study of one group could be subjected to the evaluating methods of another group and *vice versa*. Comparison of results with the same drug ought to be useful and we might be able, then, to determine the relative value of the different means of evaluation.

The use of identical protocols, particularly for the reference drugs, would certainly be helpful. It might be difficult to come to an agreement, but not only would it tell us more about what to expect of our standard drugs but also it would greatly increase the number of controls available and render a smaller concurrent control group in each study so that we could devote more of our rather limited patients to the study of new agents.

At the moment, the panel does not feel that there is a new drug worthy of incorporation in a long-term comparison in myelocytic leukemia. We are satisfied with the studies in progress. We do not feel that an untreated control group would be justified. On the other hand, some of this panel feel that in chronic lymphocytic leukemia it might be useful to have a control study of patients, untreated with alkylating agents, receiving good supportive therapy except that local X-ray or steroid therapy would be given for specific serious complications. At the moment, we do not believe that any new drug deserves to be put on a long-range trial in this disease.

We have a number of questions, one that has come up recurrently: "Has any panelist noted an increase in acute blast crises in chronic myelocytic leukemia with various means of treatment?" Our group has not. Dr. Donohue.

Dr. Donohue: I think not.

Dr. Shullenberger: I think not.

Dr. Huguley: The question was asked whether the comparative long-term studies reported were sufficiently conclusive to answer questions of difference. This, of course, is a sore question. How could they be designed to do better? In these long-term studies it is obvious that the answer is going to come only from survival data and we cannot answer that question yet. Dr. Donohue, has demecolcine been useful in cases which could not be controlled by Myleran?

Dr. Donohue: I have not had sufficient experience to answer that question.

Dr. Huguley: I started to mention that one value of the chronic leukemias is that we seldom get into a problem of the diagnosis. This is a problem with lymphomas. But some one is asking Dr. Donohue, How do you differentiate chronic lymphatic leukemia from a lymphoma with a high lymphocyte count, as far as classifying it for your study?

Dr. Donohue: We require for diagnosis that the patient have an absolute lymphocyte count of 15,000 and in addition must have adenopathy or a palpable spleen. If the white count is below 15,000, the patient is not considered eligible for treatment and can be classified at the option of the investigator as having lymphocytic lymphoma. The increased lymphocyte count must be of characteristic mature lymphocytes rather than young ones.

Dr. Huguley: The question is asked, Why is the bone marrow finding not utilized in evaluation of response to therapy in chronic leukemias?

Our group certainly does use it. Dr. Donohue.

Dr. Donohue: We do not know of a way to quantitate it accurately enough for our purpose.

Dr. Sprague: We have not used it in the chronic lymphocytic leukemia protocol. It is hard to believe that there would be any striking effect that would not be reflected in peripheral blood.

Dr. Huguley: It certainly is reflected in the peripheral blood. In our group, with chronic lymphocytic leukemia we have not defined a response as excellent unless the patient was not only lacking in symptoms and physicial findings of the disease but unless the percentage of lymphocytes in the marrow had been reduced to less than 25 percent. This puts a pretty strong demand on the drug.

Dr. Sprague, did adrenal steroids alter the score assigned to a patient at the onset of treatment?

Dr. Sprague: If patients had received steroids, at least 3 weeks must have elapsed before they were allowed to be entered in the studies. If during their course in the study they were placed on steroids and if steroids were continued for 6 weeks, they were dropped from the study as a treatment failure. So, initially on entry, they would not be entered in the study if they were on steroids or if they had received them within the 3-week period.

Investigations in Malignant Lymphomas[1]

Chairman: DR. R. WAYNE RUNDLES, *Duke Hospital, Durham, North Carolina*

Members of Panel:

Dr. Robert Lukes, *Armed Forces Institute of Pathology, Washington, D.C.*
Dr. Lauren Ackerman, *Washington University, St. Louis, Missouri*
Dr. James Holland, *Roswell Park Memorial Institute, Buffalo, New York*
Dr. Paul Spear, *Veterans Administration Hospital, Brooklyn, New York*
Dr. James L. Scott, *Wadsworth Veterans Administration Hospital, Los Angeles, California*
Dr. Charles L. Spurr, *Bowman Gray School of Medicine, Winston-Salem, North Carolina*

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[1] Presented at Conference on Experimental Clinical Cancer Chemotherapy, Washington, D.C., November 11, 1959.

Dr. Rundles: In planning this panel discussion, my colleagues and I have agreed to spend little time reviewing well-known information, but to focus our attention, for the most part, on what appear to be the key problems involved in studying the effects of chemotherapeutic agents in patients with malignant lymphomas.

In dealing with this group of diseases, we encounter at the outset many difficult, if not unique, diagnostic problems. A node removed for pathologic study may or may not be a true aliquot of the total disease. The pathologic interpretation frequently varies from one observer to another. If the biopsy technique, including the removal, fixation, sectioning, and staining of tissue, is not especially meticulous, reliable diagnostic conclusions may not be possible.

The basic pathologic characteristics of a lymphoma in a given individual at a given time, moreover, often change as the disease evolves. The usual evolution is toward decreased tissue differentiation, an increasing degree of cellular malignancy with a tendency to invade normal tissue, and/or leukemic generalization. In designing studies and interpreting experimental data, we must keep these variables in mind.

To help us discuss these and other matters we are fortunate in having two of our consulting pathologists, Dr. Lauren Ackerman and Dr. Robert Lukes, who will outline a proposed classification of the malignant lymphomas and summarize criteria they use in their identification. They will make some suggestions regarding technical problems and discuss matters requiring further investigation.

In reference to our more general problems, the members of this panel are agreed that quantitative studies of chemotherapeutic agents are both desirable and practical to carry out in patients with malignant lymphomas. The incidence of these diseases in almost all compilations exceeds that of the chronic and acute leukemias together. They represent, accordingly, a significant segment of neoplastic disease, and in almost all medical centers there are enough patients available to be studied efficiently. Most patients with malignant lymphomas present objective indexes of disease activity that can be used to evaluate therapeutic responses. The results of therapy are good enough that patients are cooperative and willing to spend considerable time and effort, if necessary, to obtain treatment. Some degree of therapeutic success seems necessary, as well as hope for the future, to maintain the morale of patients and that of their doctors.

Your panel has agreed further that the first objective of chemotherapeutic studies in these patients is to select and develop more efficient agents and regimens for their immediate care. The quantitative effects of some agents might be easier and better studied in diseases other than the malignant lymphomas. For instance, two agents that affect the proliferation of mature lymphocytes predominantly could be more easily compared in patients with chronic lymphocytic leukemia. In the malignant lymphomas there is, however, usually an abnormal proliferation of more primitive elements derived from reticulum and connective tissues. To evaluate the effect of chemotherapeutic agents on these tissues requires study in patients with malignant lymphomas, which cannot be carried out in those with any other disease. If an unsuspected degree of activity were discovered for an agent during the course of such studies, it might fortuitously lead to an advance in the control of other diseases and thus produce an additional dividend.

We should now consider the strategy and logistics of chemotherapeutic studies in the malignant lymphomas. It is axiomatic in investigative work that diagnoses must be as accurately established as possible under the best available circumstances. Terminology should be standardized, or at least well enough defined to be comprehensible to every worker in the field. Before experimental chemotherapy is started, the course of each patient's disease should be fully documented as to its rate of progression, anatomic extent, and complications.

We all agree that the malignant lymphomas evolve from less malignant to more malignant forms, and from more or less circumscribed to more generalized entities. The rate of progression and the direction of evolution of disease in any given patient are unpredictable.

Objective measurements of disease are essential. Some indexes can easily be observed and recorded. Superficial nodes, tumors, spleens, etc., can be measured by yardstick, caliper, or roentgen shadow. Determining the presence and amount of occult disease is a more difficult and perennial problem. Dr. Holland will comment later on some advances in this area.

All of us are skeptical, I think, about relying on indirect reflections of disease activity, *i.e.*, fever, anemia, and serum protein changes, as satisfactory gauges of therapeutic effect.

In evaluating responses to experimental chemotherapy, we use the same measurements as during the pretrial period to study the rate of disease progression and activity. Some time is required in any chemotherapeutic trial for the effect of an agent to become evident on the course of disease. Most of us on this panel agree that in the malignant lymphomas the trial period should be at least 2 months, even if the agent is given in full therapeutic dosage. Three months may be closer to optimal in most instances. In some entities such as Hodgkin's disease, in which spontaneous fluctuations in disease activity are common, trials that last 4 to 6 months may permit a better judgment as to the potential value of a given agent.

It is unwise to design large-scale studies before sound pharmacologic and dosage information is available for the drugs to be used. We should not try to solve problems which we are not sure exist. We have no indication, for instance, that any agent clearly inactive or inferior, when used in adequate dose for a period of 2 to 4 months, would be any more effective if it were given for a longer period. We are agreed that the maximal safe dose of all agents should be cautiously titrated for each individual patient and, when determined, be maintained for a long enough time to affect disease activity.

The suggestion has been made in this symposium that better results might be achieved by giving patients with malignant disease submaximal doses of different agents. We know of no instance in clinical cancer chemotherapy in which a therapeutic prozone has been demonstrated. Until such a phenomenon is shown to exist, we should not complicate our studies, slow and laborious as they already are, by considering probably irrelevant biologic parallels.

The number of patients required for different phases of chemotherapeutic study will determine the number of compounds we can investigate. We will return to this matter of sample size later.

The pathologic classification and grouping of the malignant lymphomas has been extensively, and in part fruitlessly, discussed for many years. It is probable that only now can we really assemble definitive information. Our Pathology Advisory Committee proposed some months ago the following classification and grouping:

Malignant lymphomas
Provisional classification and grouping

1. Follicular lymphoma
2. Lymphocytic lymphoma
3. Lymphoblastic lymphoma, lymphosarcoma (large-cell), reticulum-cell sarcoma
4. Hodgkin's disease(s)
    - Paragranuloma
    - Granuloma
    - Sarcoma
5. Malignant lymphomas, unclassified

("It is better to submit to a judicial decision, even though arbitrary and possibly erroneous, than to have endless controversy."—C. Northcote Parkinson.)

This is intended to be provisional, and subject to long-term verification on the basis of future experience. Dr. Robert Lukes will at this time outline some problems pathologists face in diagnosing and classifying the malignant lymphomas.

Dr. Lukes: The proposed classification of malignant lymphomas is simple, but there are hidden problems which will make valid comparisons of the effectiveness of different chemotherapeutic agents very difficult—technical factors, the possibility of error in diagnosis, and differences in terminology and classification. Since the validity of this study is greatly dependent on the accuracy of the histologic diagnosis, I would prefer to emphasize certain technical and diagnostic problems facing the pathologist and not readily apparent to the clinician, rather than the intricacies of classification and terminology.

From textbook descriptions it would seem to be an easy matter to identify and classify a malignant lymphoma. The proliferative lesions of lymphoid tissue, however, are regarded by pathologists as one of the most difficult fields of histologic diagnosis. Figures 1 and 2 demonstrate the possible cytologic similarity of a malignant lymphoma and a benign lymphoid lesion, and the importance of excellent histologic sections for accurate interpretation. The lesion in figure 1 is a malignant lymphoma of well-differentiated lymphocytic type, and the other (fig. 2) is a severe benign lymphocytic proliferation in chronic lymphadenitis. The differentiation of these lesions may be difficult, if not impossible, on improperly fixed tissue or on thick, deeply stained sections. As a result of faulty technical preparation of tissue and the possibility of severe reactive processes simulating malignant lymphoma, benign lesions may be readily misinterpreted as malignant lymphoma, unless particular caution is exerted in the study of lymphoid tissue lesions.

I would urge that special attention be given to the selection, biopsy, and handling of lymph nodes before they reach the pathologist.

Selection of the node for biopsy is an important factor in the accurate histologic evaluation of case material. The clinician should carefully identify the most significantly enlarged lymph node, usually the deepest and largest one, since this type will probably demonstrate the most characteristic involvement. The surgeon should be thoroughly familiar with the specified node and mark it before surgical drapes obscure the field. Biopsy of the most accessible or smallest node should be avoided, as this leads to unrepresentative biopsy material and undoubtedly accounts for the discrepancy that occurs at times between the clinical and the histologic findings.

The node should be carefully removed by sharp dissection. Particular caution should be exerted to obtain an intact node, avoiding pulling and fragmentation, because evaluation of the architecture is essential for histologic diagnosis.

The unfixed nodes should be taken from the surgeon to the pathologist immediately after removal. The pathologist should cut them at once into tissue blocks 2 to 4 mm. in thickness. Before the blocks are dropped in the fixative, imprint ("touch") preparations should be made, since they provide invaluable assistance in the study of cytologic detail. Unfortunately, it is a common practice to drop the entire lymph node into the fixative, frequently after considerable time has elapsed. If the node is sizable, or if it is not sectioned until many hours later, delayed penetration of the fixative occurs, which results in marked cytologic distortion in the central portion and considerable difficulty in interpretation. It is my belief that the fixative selected is not nearly as important as is the preparation of the tissue for the fixative. In my experience, buffered 10 percent formaldehyde has proved to be a satisfactory fixative for lymphoid tissue when the tissue is properly handled.

Particular attention and skill are essential for the preparation of histologic sections of lymph nodes. Thin histologic sections no more than one cell thick (6μ), lightly stained with hematoxylin and eosin to permit appreciation of both cytoplasmic and nuclear detail, are required for diagnostic evaluation. The significant cytologic features of Reed-Sternberg cells of Hodgkin's disease, particularly the characteristic large inclusion-like nucleoli, are commonly obscured in the excessively thick and deeply stained histologic sections usually provided for study. Although it is well known that Reed-Sternberg cells occur in mononuclear, lobated, binucleated, and multinucleated forms (figs. 3, 4, 5, and 6), the best known of which is the classic mirror-image cell (fig. 5), the significance of the large inclusion-like nucleoli is not generally appreciated, since they are not distinct in deeply stained sections. The essential requirement for an accurate histologic diagnosis of Hodgkin's disease is the Reed-Sternberg cell with more than a single nucleus and with the large inclusion-like nucleoli. To obtain the most ideal sections consistently for accurate diagnosis, the Southeastern group employs one specially trained technician to prepare all the histologic sections.

In addition to the technical factors, the pathologist must be constantly aware of the remarkable capacity of the reticuloendothelial system to react in disease and simulate malignant lymphoma. The lymphocytic proliferation in infectious mononucleosis provides one of the most dramatic examples of this situation (fig. 7). In this process the lymph-node architecture is severely altered and, at times, almost completely obliterated. The lymphatic follicles are infrequent or absent and there is prominent infiltration of the lymph-node capsule, which presents a histologic lesion readily misinterpreted as malignant lymphoma. The cytologic features of the abnormal lymphocytes may be identified in very well-prepared sections (fig. 8) but obscured in those imperfectly done.

Figure 9 presents an example of severe follicular hyperplasia of a lymph node, a lesion that is commonly confused with follicular (nodular) lymphoma. This lymph node is from a patient with rheumatoid arthritis, a condition in which severe follicular hyperplasia is rather characteristic. It is often seen in patients who have generalized enlargement of lymph nodes, frequently with splenomegaly and hematologic abnormalities, and who present a clinical situation appropriate for the diagnosis of malignant lymphoma. Severe follicular hyperplasia, unassociated with other evidence of disease, also occurs and presents one of the most difficult differential diagnostic problems for pathologists. In the study of the large volume of case material received in the Lymphatic Tumor Registry at the Armed Forces Institute of Pathology, I have encountered a wide variety of severe benign reactions of lymphoid tissue that markedly alter the lymph-node architecture and often closely mimic malignant lymphoma. From this experience I believe that the pathologist must be constantly aware of the possible simulation of malignant lymphoma by a reactive process.

Through the special technical handling of biopsy material now employed by the Southeastern group at the Armed Forces Institute of Pathology, and with the awareness of the extraordinary reactive capacity of the reticuloendothelial system, I believe we can provide a reliable objective basis for the diagnostic evaluation of lymphoid-tissue lesions. In the Southeastern group, the sections are studied independently by Dr. Lauren Ackerman, Dr. Fred Collier, and myself. The final diagnosis represents a combined opinion.

We shall now turn to a brief consideration of our provisional classification of the malignant lymphomas, in which we have adopted 5 major types. There are also a number of morphologically distinctive subtypes within these major areas that may be of considerable importance in this study. Although we are at the present time classifying the lesions only according to the major type, it will be necessary to determine eventually whether the important differences in evolution of disease are related to the response to therapy or to these morphologic subtypes. Hodgkin's disease represents a well-known example in which the subtypes described by Jackson and Parker are generally considered to have rather distinctive natural histories. At this time I merely want to emphasize that the final analysis of the results of the effectiveness of chemotherapy must take into consideration the possible significance of these subtypes of malignant lymphoma.

To summarize, in the histologic study and interpretation of lymph nodes removed from patients with clinically suspected malignant lymphoma, there are a number of problems that concern the pathologist but may not be apparent to the clinician. Accurate morphologic diagnosis and classification have a critical bearing on the evaluation and comparison of the results of chemotherapy. In order to achieve this goal, it is essential that special attention be given to the selection of the lymph node, the biopsy technique, and the processing of the tissue. Finally, if different groups of investigators plan to compare their chemotherapeutic results, I believe it will be essential for the pathologists of each study group to collaborate in a comparative study of histologic case material in an attempt to achieve uniform criteria for diagnosis and classification of lymphomas.

Dr. Rundles: I would like to ask Dr. Ackerman if he can give us an estimate as to how frequently errors occur in the diagnosis of the malignant lymphomas. Are they frequent enough or misleading enough to affect materially the accuracy of large-scale studies?

Dr. Ackerman: Pathologists as a group, like hematologists and chemotherapists, are improving. In the lymph-node area, as in the realm of bone pathology, there are very difficult problems of diagnosis. The two problems of overcall and undercall are serious. If you combine these with a situation in which there is a pathologist who will look at and interpret very thick slides, about the only thing that I can predict is trouble. This situation could easily destroy the value of any study. It is imperative that we have nodes to study that are well selected, that sections be well prepared and suitable for accurate interpretation.

Dr. Rundles: Some months ago Dr. Ackerman told us that if we were going to make mistakes, we should make them on a high plane. I would like to call your attention now to one of our problems which has resisted high-level study.

While we always attempt to classify the lymphomas as to type and subgroup, practical therapy can usually proceed if we can only be sure that the patient just has some type of lymphoma. It is not too infrequent, however, that despite our best efforts, we cannot decide whether we are dealing with an unusual lymphoma or with an undifferentiated carcinoma.

The example I would like to cite is a 55-year-old colored farmer's wife, who in the early part of 1959 began to cough, have anorexia and night sweats. Her symptoms fluctuated and in April of this year she began to lose weight from her average of 180 to eventually only 134 pounds. Finally on July 29, 1959, she was admitted to the hospital, complaining of shaking chills, fever, shortness of breath, and edema. On examination she was acutely ill, poorly nourished, weak, and apathetic. Her temperature ranged up to 38 to 39° C. There were many large, superficial lymph nodes in the cervical areas and axillae. Masses were present bilaterally in the lower abdomen. Her hemoglobin concentration dropped from 6.2 to 5.4 gm. percent during the first few days she was in the hospital. Her white blood count remained elevated to between 22,500 and 28,875. There were no abnormal cells in the circulating blood. Her bone marrow was very cellular and otherwise normal.

After a blood transfusion had been given we were able to get a lymph-node biopsy. The node imprints contained bizarre cells varying greatly in size and morphology. Nucleoli were prominent and there were many mitotic figures. Some of our pathologists, after studying the imprints as well as the fixed sections, favored the diagnosis of Hodgkin's disease, while others thought she had undifferentiated carcinoma. In view of the split vote, we asked for the opinion of our Committee on Pathology. After a second review, the vote was still split: Two were in favor of undifferentiated carcinoma and one was for Hodgkin's disease.

Now we would ordinarily say, what difference does it make? Wait, and the autopsy will probably clarify the situation. It happens, however, that this particualr individual, along with several others in our current generation of patients, is not in the least interested in an autopsy.

She was treated with Cytoxan and given what we consider to be a maximal safe amount initially, a total of 40 mg. per kg. intravenously, divided into 4 doses. After the third injection, she became afebrile and her nodes began to disappear. By August 19, 1959, 2 weeks after her discharge from the hospital, she had resumed working on the farm. Her weight had stabilized and her hemoglobin concentration had started to rise.

The Cytoxan chemotherapy was maintained on an intermittent schedule. Ten weeks later, on return visit, her performance status was rated at 100 percent. On examination she had no palpable enlarged nodes or tumor masses. Her weight had risen to 151 pounds. Her hemoglobin concentration was 11.8 gm. percent with all other hematologic values normal. X-ray films of her chest showed that the evidences of disease present before therapy, enlarged hilar nodes and widening of the mediastinum, had disappeared completely.

If this were an isolated case it would not be especially important. We have a second individual who has done equally well, however, in whom we have been unable to differentiate carcinoma from lymphoma. In this connection, 6 out of 20 patients, who we are satisfied have undifferentiated metastatic carcinoma, have shown objective responses to Cytoxan, which have now been maintained for as long as several months. Five consecutive patients with reticulum-cell sarcoma have responded to an unusual degree to this agent, in some instances after other mustard compounds had apparently not been effective.

Our conclusion from this experience is that we must be careful that certain pathologic diagnoses do not deprive our patients of potential benefit from new modes of therapy. We cannot be sure, either, that good therapeutic responses to a given agent provide any certain indication as to the source or type of a tumor. While we investigate in more detail the relationship of pathologic and biochemical features of tumors to their therapeutic response, we must take considerably more interest in the problems our pathologist colleagues have in handling and interpreting tissue biopsies.

We shall now consider the objective measures of disease activity that can tell us how sick a patient is and to what extent he may have responded to a course of experimental chemotherapy. We all use the same general methods in quantitating obvious disease: node and spleen measurements, X rays of chest and soft parts, fever, anemia, etc. In reference to detecting the presence of and measuring the amount of occult disease, some new developments in the field of lymphangiography will be reviewed now by Dr. Holland.

Dr. Holland: The Eastern solid tumor group which I represent has put much effort into studying measurement techniques and methodology. We have been reluctant to accept indirect measures of disease activity or codifications similar to some that have been presented. As a matter of fact, one of our accomplishments may be the reporting of an occupational disease known as "caliper abrasion," from the number of thumbs traumatized by calipers during the measurement of masses in subcutaneous tissues and nodes.

In the field of the lymphomas, we have all had difficulties in measuring tumors in the retroperitoneal areas. I feel somewhat like an interloper in discussing a diagnostic radiologic technique for the demonstration of retroperitoneal lymph nodes. I wish to make it clear that my colleagues, Dr. Myzoslaw Hreshchyshyn and Dr. Richard Shehan, have done this work. I have selected the patients, encouraged them to have the studies, then encouraged the doctors, and finally looked at the films. Since Dr. Hreshchyshyn is here, he would be the proper one to whom to address questions regarding technology and techniques in the performance of these studies.

Figure 10 shows the presence of ethiodol in retroperitoneal lymph nodes after its injection via a lymphatic on the dorsum of the foot. This lymphatic is first identified by the intracutaneous injection of blue dye. Dr. Hreshchyshyn's skill allows him to pick out and canulate this lymph vessel, and introduce ethiodol. These roentgenograms, a posteroanterior view and oblique, are of a 30-year-old woman with Hodgkin's disease who had pruritus. The question arose as to whether this symptom was indicative of retroperitoneal adenopathy. Although we have not actually studied normal individuals, the nodes demonstrated in her pelvis and in the paravertebral areas are apparently normal in size. This patient had had an exploratory operation a short time previously, and nodes were not seen in this area when the initial biopsy was made.

Figure 11 shows enlarged lymph nodes in a man with chronic lymphocytic leukemia. Another area of involvement is shown in a 22-year-old boy who had a large mass of nodes arising deep in the pelvis (fig. 12). The remainder of his retroperitoneal nodes were not definitely enlarged. He was treated with a course of thio-TEPA, following a protocol which we were using, and the oily, radiopaque material retained in the lymph nodes then showed a change in the size of the mass. Since this agreed with the finding on abdominal palpation, we have a measurement technique translatable into standard units such as centimeters, which confirmed the therapeutic effect of thio-TEPA on this boy's tumor.

The next roentgenograms, taken 2 months later and then at 3 months, showed the nodes to have again increased in size, slightly but definitely. At this point we deemed it necessary to interrupt his thio-TEPA therapy, inasmuch as we had obtained objective evidence of progression in his disease. Since he was seriously ill at that time, he was treated with corticosteroids. After some hematologic and clinical improvement, HN2 was administered. Again there was a decrease in the size of nodes in this deep abdominal area (fig. 13). The injections of dye were not repeated, since the contrast medium injected earlier was still retained in the lymph nodes.

The converse of this situation can be illustrated by another patient (fig. 14) who had lymphosarcoma with an area of bone involvement that resulted in a pathologic fracture. Some weeks previously lymphangiography had demonstrated large lymph nodes in this area. She was treated with methotrexate or dichloromethotrexate in a study which Dr. Spurr will describe. In the course of this experimental chemotherapy, roentgenograms showed a decrease in the density of dye and an increase in the mass of lymph nodes. This then was objective evidence of progression of disease during the study. In this instance the measurement technique helped us in the management of the patient.

Dr. Rundles: We obviously need to use these and all other available aids in making objective measurements to help us evaluate better what we do or do not accomplish in experimental chemotherapy.

We shall now consider what categories of agents we know about that are worth quantitative evaluation in the malignant lymphomas. Before we choose 2 or 3 dozen likely candidates to investigate by the end of this year, though, we might well consider the logistics involved. How many patients will we have to study to (1) demonstrate that a given agent has some degree of activity in a specific disease, (2) exclude a significant degree of activity, or (3) compare the relative efficiencies of 2 active agents?

As Dr. Lasagna pointed out, the number of patients required for different phases of study will depend on the potential responsiveness of their disease to therapy, and how well the subjects are selected. We usually learn little or nothing from treating patients who are approaching a terminal status, or who are hopelessly complicated by disease or previous treatment when chemotherapy is first contemplated. As cancer chemotherapy gains in stature, many more suitable candidates are becoming available for study.

To show that a new agent has some degree of antitumor activity in the human, most of us would choose to study its effect first in patients with responsive diseases, leukemia or some type of malignant lymphoma. If activity were demonstrated, quantitative evaluation would then obviously be in order. If, during exploratory trials, a new agent were to produce one unequivocal regression in the growth of a tumor ordinarily unresponsive or rarely responsive, such as a hypernephroma, melanoma, alveolar ridge or other type of carcinoma, it would be highly significant.

A common problem, though, is to exclude activity of an agent in one or more tumors in the human. All too frequently a new compound is given to 2 or 3 patients, and when no obvious effect is produced, it is discarded. To establish a reliable negative result requires more work than we ordinarily put into it. As we shall see, it requires more than 20 consecutive failures to exclude the possibility that the compound has an activity of even 10 percent. Most hospital groups lose interest very rapidly after 4 or 5 failures, at the most, so reliable negative findings require interinstitutional cooperation.

In a discussion of this matter of negative results with our consulting biostatitician, Dr. James Grizzle, it appears that we can calculate the number of trials required if we make assumptions regarding the assumed activity of a drug and are willing to specify the accuracy with which we hope to work (table 1). It will require 16 consecutive failures to show with 80 percent confidence that a drug has antitumor activity in less than 1 patient in 10 with a given disease. Twenty-one consecutive failures will be required to increase our confidence to 90 percent. To show an activity below 20 percent would require half as many patients, and an activity of 40 percent only one fourth as many.

Table 1.—Sample size in chemotherapy trials

Number of *consecutive* failures required to exclude activity:

Assumed activity of drug (*percent*)	80% Confidence	90% Confidence
10	16 patients	21 patients
20	8 "	11 "
50	3 "	4 "
80	2 "	3 "

Number of patients required for comparison of 2 agents to show a 20 percent or greater difference in therapeutic efficiency:

	Frequency of remission (*percent*)	80% Confidence	90% Confidence
Early cases	80	60 patients (each agent)	80 patients (each agent)
All cases	60	95 patients	125 patients
Late cases	50	100 patients	130 patients

If we want to design a study to compare the therapeutic efficiency of 2 agents with fairly good activity, a surprisingly large number of patients is again required (table 1). We can estimate that around 80 percent of all patients with early or previously untreated malignant lymphomas would respond to our best presently available chemotherapeutic agents. To compare the efficiency of 2 of them and show that a 20 percent difference in therapeutic efficiency exists, we would have to study the effect of both agents in 60 patients, providing we are willing to be satisfied with an 80 percent confidence limit. For 90 percent confidence, 80 patients would have to be studied. If the patients we selected to study had advanced disease, of whom about half would respond to therapy, 100 patients would be required for 80 percent accuracy, and 130 for 90 percent accuracy.

It is apparent from these logistic considerations that the agents we select for definitive studies should be carefully chosen.

Several classes of agents have given therapeutic responses in patients with malignant lymphomas, and provide us with leads for further study. These include irradiation, the nitrogen mustard compounds and derivatives, and adrenocorticosteroids.

In reference to irradiation, most of us agree with Dr. Ravdin that we cannot anticipate tremendous advances from this modality, even with the development of supervoltage apparatus and other technical improvements. Nevertheless, irradiation is still the standard mode of therapy for localized lymphomatous disease. Our panel has discussed at some length whether or not any harm would result from the postponement of local irradiation therapy in such an individual with localized disease for a 2- to 4-month trial of chemotherapy. If local disease were to persist, irradiation would then, of course, be given. At the risk of making our radiologists feel rejected, I should like to say that it is my minority opinion on this panel that such a period of delay to study the effects of any active chemotherapeutic agent would not jeopardize the patient's future. Many clinicians, however, will prefer to go ahead with irradiation immediately, even though it would surely retard the progress of chemotherapeutic studies. In my opinion, our patient clientele has so much more at stake in our work that expedient palliation has little justification.

Another question that is often asked is, Has chemotherapy utilizing the nitrogen mustard compounds replaced spray or whole-body irradiation in the treatment of the malignant lymphomas? The vote of this panel is at least 4 to 1 that it has. Most of us see few or no indications now for whole-body irradiation.

The nitrogen mustard compounds and their derivatives of definite value in the treatment of the malignant lymphomas are numerous. They include HN2, thio-TEPA, Nitromin, TEM, chlorambucil, phenylalanine mustard, uracil mustard, Cytoxan, etc.

Myleran, a different type of alkylating agent, should be mentioned parenthetically. I should like to make it clear what our position is in reference to this agent. We are not among those who think that all "alkylating agents" are the same. We believe that we have demonstrated a clear-cut difference between the antileukemic and hematopoietic effects of Myleran and chlorambucil when assayed in patients with chronic lymphocytic and granulocytic leukemia. Our findings were reviewed by Dr. Huguley earlier. We are 99 percent sure that there is a significant difference in the tissue specificities of these 2 agents and we do not plan to study the matter further. We do not intend to study the effect of Myleran in the malignant lymphomas.

Now in reference to the comparative effects of nitrogen mustard, thio-TEPA, and Nitromin, two groups have conducted relevant studies. I would like to ask Dr. Holland if he will summarize the experience of the Eastern group who compared the effects of nitrogen mustard with those of thio-TEPA.

Dr. Holland: In this study there were relatively few patients—considerably less than the number of investigators, I think. We selected patients with untreated Hodgkin's disease and on a randomization basis assigned them to groups for treatment with either nitrogen mustard or thio-TEPA. Their response was judged by a voting procedure which Dr. Chalmers described in reference to lung cancer. All 7 of the patients treated with nitrogen mustard responded, but only 8 of the 11 treated with thio-TEPA (text-fig. 1). The duration of response after nitrogen mustard therapy lasted longer, but we were surprised in studying the life tables of these patients to see that those who received thio-TEPA seemed to live a little longer. The data were not, however, significantly different. The initial drop in white blood count in each group was equal, but the patients treated with thio-TEPA had a final white blood count of 5,000, whereas those treated with nitrogen mustard had a median white blood count of 2,000. For this reason we thought that perhaps the doses we gave of the 2 compounds were not strictly equivalent.

EASTERN SOLID TUMOR GROUP

STUDY I

	HN2		TSPA	
	0.05	0.1	0.2	0.4
"Success" / Total Treated		7/7	8/11	

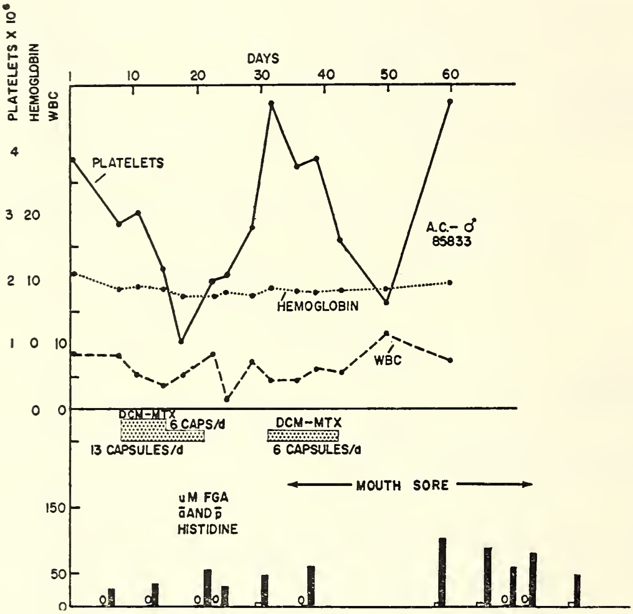
STUDY II
(Incomplete 1959)

	HN2		TSPA	
	0.05	0.1	0.2	0.4
"Success" / Total Treated	1/4	3/4	1/5	3/4

TEXT-FIGURE 1.—Chemotherapy of Hodgkin's disease. Results in patients receiving their first course of an alkylating agent.

We then undertook a second study, which is still in progress, using 2 dose levels of thio-TEPA. The doses were chosen to investigate whether the observed differences were drug specific or dose specific. On one schedule thio-TEPA was given in a dose of 0.4 mg. per kg. for 4 successive days, followed by a 2-week rest period, and then 0.4 mg. per kg. given weekly for 90 days. This was intensive alkylation therapy. Another group was treated with half this amount of drug given at the same interval. On the low-dose regimen, remissions were produced significantly less frequently than with the higher doses. We think we have shown, to our satisfaction at least, that with the low-dose regimen there is a dose-response curve, but that we were too low on it to produce good results. This, I think, is a point in favor of Dr. Rundles' statement earlier that we have not recognized in cancer chemotherapy a prozone phenomenon. In the group of patients treated with the higher doses of thio-TEPA and with the standard dose of nitrogen mustard, there were 3 out of 4 remissions with each agent. We are continuing this study further, hoping to shed some light on whether or not there is specificity among the alkylating agents, or whether, if they are given in biologically equivalent doses, one might produce identical results.

Text-figures 2 and 3 illustrate a study that we are pursuing at Roswell Park, in which patients on the folic acid antagonist study have been examined for formiminoglutamic acid excretion in urine before and after histidine challenge. Text-figure 2 gives data on a man with lymphosarcoma who had a transient tumor response while receiving the full dose of folic antagonist drug (I do not know what the drug was or what the dose was since we do not break the code). There was a minor decrease in his tumor masses and he felt slightly better, while there was little change in his formiminoglutamic acid excretion. Long after the drug was stopped, a maximum occurred at which time he had developed prolonged toxicity in the mouth.



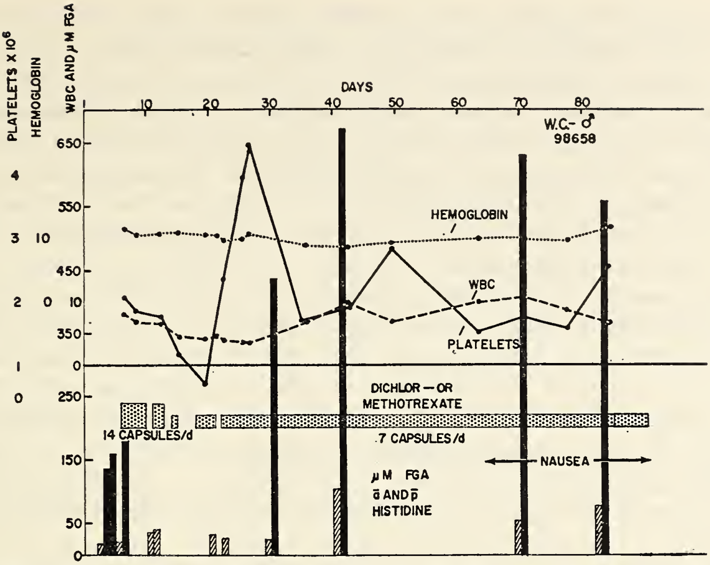
Text-figure 2.—Study of a patient with lymphosarcoma who had a transient tumor response while receiving the full dose of folic antagonist drug. At bottom, *open circle*=formiminoglutamic acid excretion without the histidine load; *solid bar*= patient received 5 gm. of histidine that day.

These findings demonstrate that folic acid antagonists have long-lasting effects. They may not be studied in the concept that, while one gives the drug, one expects to see the chief effects. Here also, is the interesting separation of antitumor effects from others. This patient's tumor had regrown by the time of the cessation of drug. There was temporal separation of the thrombocytopenic effect, of mouth toxicity, and of a biochemical parameter.

Text-figure 3 gives data on a man who had lymphosarcoma and basal-cell carcinomas—2 on the side of the face—and was treated with folic acid antagonists.

He had a remarkable and substantial remission of his lymphosarcoma and was apparently cured of the basal-cell carcinomas, in that there was no evidence of them afterward.

He developed clinical toxicity only at the very end of the course of treatment. There was no biochemical correlate of this toxicity because



Text-figure 3.—Study of a patient with lymphosarcoma and basal-cell carcinomas, who was treated with folic acid antagonists. At bottom, *shaded bar*=the formiminoglutamic acid excretion without the histidine load; *solid bar*=patient received 5 gm. of histidine that day.

his inefficiency in metabolizing formiminoglutamic acid after histidine load had been present for many weeks.

We are continuing to explore the interrelations of folic acid antagonists on formiminoglutamic acid metabolism and tumor suppression in clinical toxicity.

Dr. Rundles: The Veterans group has finished a study in which they compared the therapeutic effects of nitrogen mustard and Nitromin. Dr. Spear, we are interested in 2 questions: How do these 2 compounds compare in therapeutic efficiency, and is there a significant difference in their side reactions?

Dr. Spear: Our group has compared the effects of nitrogen mustard with Nitromin in 141 patients. Included in this group were patients with bronchogenic carcinoma referred to by Dr. Close. Only 61 of the patients had malignant lymphomas. As to the comparative efficiency of the 2 drugs, we found that nitrogen mustard and Nitromin were about equal. Since most of our patients had an advanced stage of disease, the over-all remission rate with both agents was about 50 percent. There was no statistically significant difference between the effect of the 2 compounds.

There did seem to be, however, a pronounced difference in the toxic reactions produced. Although we had difficulty defining what we meant by "toxicity," Dr. Arthur Glucian studied and reviewed all the records in an attempt to evaluate the side effects and reactions uniformly. We found that we could not decide what was a toxic reaction.

The interesting thing about Nitromin therapy was its association with psychotic reactions. In our patients there were 12 well-documented reactions of this type which ranged from varying degrees of confusion, bordering on stupor, to coma in some instances. Even these phenomena, unfortunately, present some difficulty in diagnosis and interpretation. Everyone is aware that in the use of a new drug, reactions that occur are likely to be attributed to it. When you are using a well-known drug, such as nitrogen mustard, the tendency is to attribute the reaction to the basic disease or to other medications that may have been used, and not to the agent itself. There may have been instances where confusion occurred in patients with terminal stages of lymphomas when treated with nitrogen mustard, which was not attributed to the chemical, but which, if it had occurred while the newer agent was being used, would have been attributed to Nitromin. Nevertheless, in allowing for all this, we had 12 well-substantiated psychotic reactions associated with Nitromin therapy.

Our conclusions, therefore, are that Nitromin has no greater therapeutic efficiency than nitrogen mustard but that it is associated with the production of considerably more psychotic reactions.

Dr. Rundles: We have very little information to present on the quantitative therapeutic effects of the orally absorbed nitrogen mustard derivatives in malignant lymphomas. Data regarding TEM, chlorambucil, phenylalanine mustard, uracil mustard, and Cytoxan would be of interest to all of us, but our only information pertains to chlorambucil at this time. The Western group designed an experiment to show what influence chlorambucil might have on prolonging remissions, in Hodgkin's disease, produced by the administration of nitrogen mustard. Dr. Scott, would you comment on your findings?

Dr. Scott: Our Western group study was limited to patients with Hodgkin's granuloma. All of them had measurable evidence of disease, enlarged nodes, hepatomegaly or splenomegaly, and usually more than one area was involved. They had accordingly moderate to relatively far-advanced disease.

We gave a standard initial dose of nitrogen mustard, 0.4 mg. per kg. intravenously, and their response was evaluated after 3 to 6 weeks. The patients that did respond to the nitrogen mustard were divided at random into 2 groups. To one we gave chlorambucil in a dose of 0.2 mg. per kg. daily, and to the other inert tablets. The duration of remission was then ascertained. With this dose of chlorambucil, we found that continuous therapy was rarely possible. The largest dose possible was given, nevertheless, until leukopenia developed, and then the dose was maintained as tolerated.

Although our data are still preliminary we can say that just about half our 88 patients with Hodgkin's granuloma responded to the initial dose of 0.4 mg. per kg. of nitrogen mustard. In those who did respond and were subsequently studied as to the duration of their remission, we can now evaluate the course of 13. The average duration of remission after the administration of nitrogen mustard alone was 11 weeks. The average duration of remission in the group treated with chlorambucil was almost exactly twice that, or 23 weeks. This difference in the duration of remission is significant for the number of patients who have completed study, but we should point out that there was a wide scattering in the length of remission. In the group given inert material the duration of remission lasted from 5 to 51 weeks. In the group given chlorambucil, the range was from 7 to 52 weeks.

Dr. Rundles: At the moment there is widespread interest in the possible effects of another nitrogen mustard compound in the malignant lymphomas, Cytoxan. Sporadic good results have been reported, but definite comparisons are still to be made.

Dr. Charles Spurr, working with the leukemia B group, has started an investigation of the effects of antifolic acid compounds in the malignant lymphomas. Dr. Spurr, would you tell us how you got this idea?

Dr. Spurr: This project actually rose from some of the findings of Dr. Abraham Goldin and others at the National Cancer Institute.

Early work on the chemotherapy of the lymphomas concerned, for the most part, a number of alkylating agents. After reviewing this work, we tended to agree with Dr. Baker that new carriers were needed to gain specificity or, to paraphrase Dr. Karnofsky's remarks, perhaps new alkylating groups.

Among other classes of chemotherapeutic agents that might be considered are antibiotic derivatives and the antimetabolites. Among the antibiotic derivitives actinomycin D should be mentioned, and 6-diazo-5-oxo-norleucine (DON). The latter, however, in a study recently completed by the Veterans group, proved to be a mediocre compound. For our studies we were more attracted to the folic acid antimetabolites.

Earlier in this conference Dr. Freireich reviewed the work of Dr. Goldin in which he demonstrated that the mean survival time of mice with L1210 leukemia was significantly prolonged by injections of dichloromethotrexate as compared to that with methotrexate or amethopterin treatment. The dichloromethotrexate, as mentioned, was given parenterally.

On the basis of this finding, Dr. Frei and his colleagues at the National Cancer Institute undertook to study the effects of dichloromethotrexate in man. Among the diseases in which investigation of the compound seemed to be indicated were the malignant lymphomas.

A survey of the older literature meanwhile revealed some reports of patients with Hodgkin's disease and other malignant lymphomas who had been treated with antifolic acid compounds. While most of them were terminal patients, and in many the dosage schedule far from optimal, enough improvement was observed on occasion to encourage further investigation.

On this basis a study was begun, using patients with Hodgkin's disease and other lymphomas. Suitable patients were recruited and assigned to groups at random so that approximately 40 percent of them were treated with methotrexate, 40 percent with dichloromethotrexate, and 20 percent with nitrogen mustard. The dose schedules chosen for dichloromethotrexate were 0.375 mg. per kg. and 0.75 mg. per kg. daily by mouth, and 0.075 and 0.15 mg. per kg. for methotrexate. Nitrogen mustard was given intravenously in a dose of 0.4 mg. per kg. on the 1st day and, beginning 2 weeks later, 0.1 mg. per kg. at intervals of 1 week.

The antifolic acid compounds were administered "blind" without either the investigator or the patient knowing which compound was given. Forty patients are now under study. We have not yet broken the code, but do know that remissions have occurred in patients treated with the folic acid antagonists.

We now have the additional information from Dr. Goldin that the effectiveness of dichloromethotrexate and methotrexate differs only when the drugs are given parenterally. Our present plans are to terminate our present study when 50 patients have been treated, and then change our tactics to study the differential effect of these compounds when they are given parenterally.

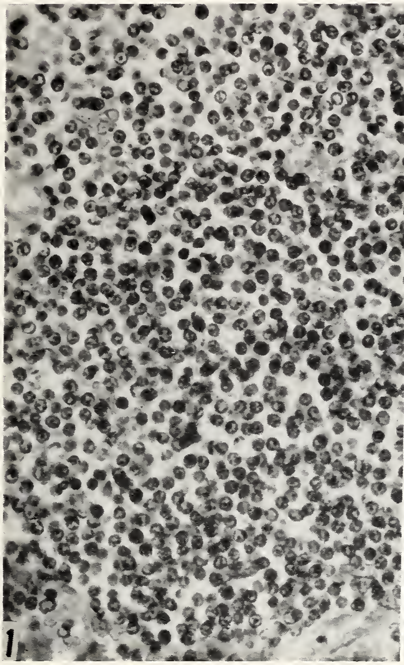
Dr. Rundles: This has been a very rapid once-over of some of the problems and projects dealing with the chemotherapy of malignant lymphomas. Many of your questions have not been answered, and many major problems await solution and discussion at future conferences.

PLATE 4

Comparison of lymphocytic lymphoma and benign reactive lymphocytic proliferation. Hematoxylin and eosin. × 400 (AFIP Neg. No. 59-2847.)

FIGURE 1.—Malignant lymphoma, lymphocytic type, well-differentiated, of lymph node. (AFIP Acc. No. 825026.)

FIGURE 2.—Benign proliferation of lymphocytes in chronic lymphadenitis. (AFIP Acc. No. 796880.)



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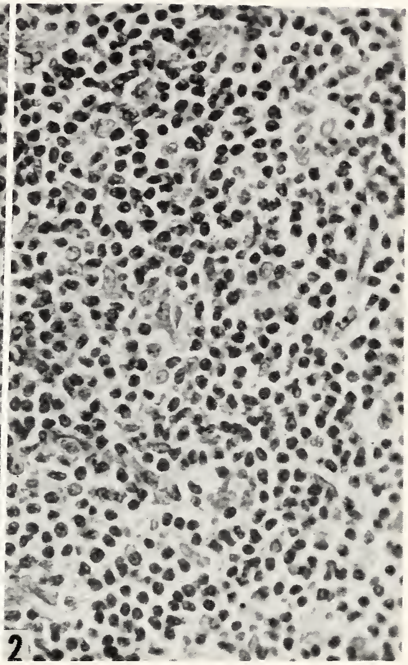

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PLATE 5

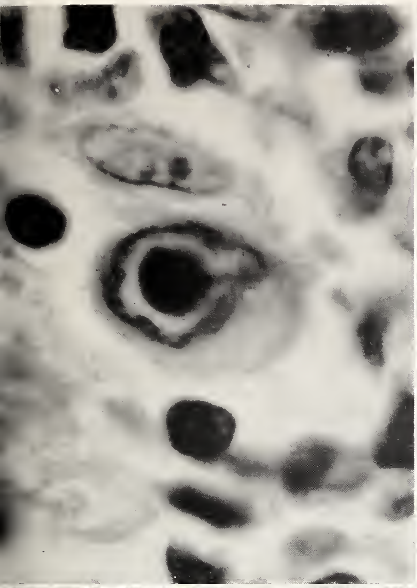
Variations in Reed-Sternberg cells. Hematoxylin and eosin. × 1,000 (AFIP Acc. No. 924516, Neg. Nos. 60-166 through 69-169.)

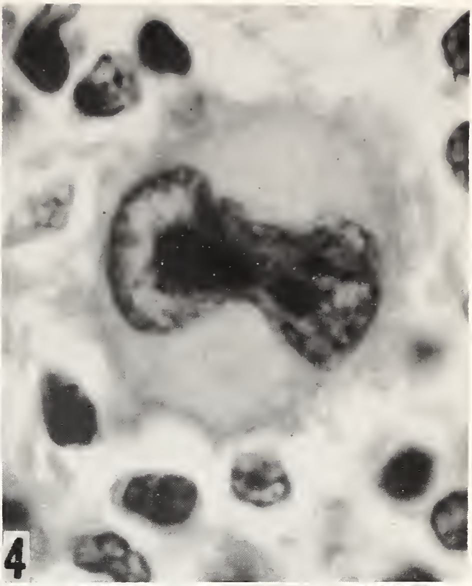
FIGURE 3.—Reed-Sternberg cells in mononuclear forms with characteristic inclusion-like nucleoli.

FIGURE 4.—Reed-Sternberg cells in lobated forms with characteristic inclusion-like nucleoli.

FIGURE 5.—Reed-Sternberg cells in mirror-image forms with characteristic inclusion-like nucleoli.

FIGURE 6.—Reed-Sternberg cells in multinucleated forms with characteristic inclusion-like nucleoli.





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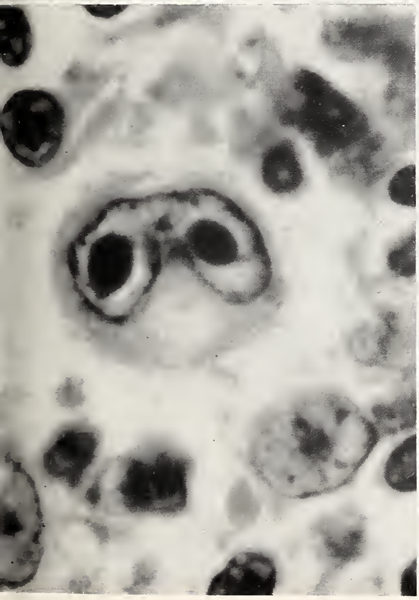

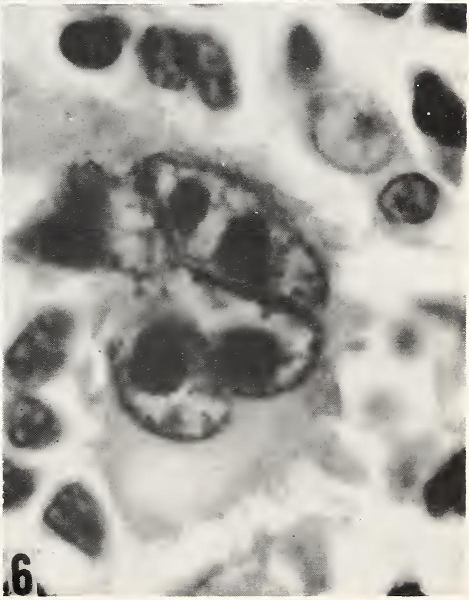

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Plate 6

Figure 7.—Lymph node in infectious mononucleosis closely resembles malignant lymphoma, since lymphatic follicles are absent, capsule is infiltrated, and architectural pattern of lymph node is obscured. Hematoxylin and eosin. × 40 (AFIP Acc. No. 663853, Neg. No. 57-1936.)

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Plate 7

Figure 8.—Atypical lymphocytes of infectious mononucleosis in lymph node. Cells in properly prepared sections resemble Downey cells of peripheral blood. Hematoxylin and eosin. × 2,000 (AFIP Acc. No. 570504, Neg. No. 59-4873.)

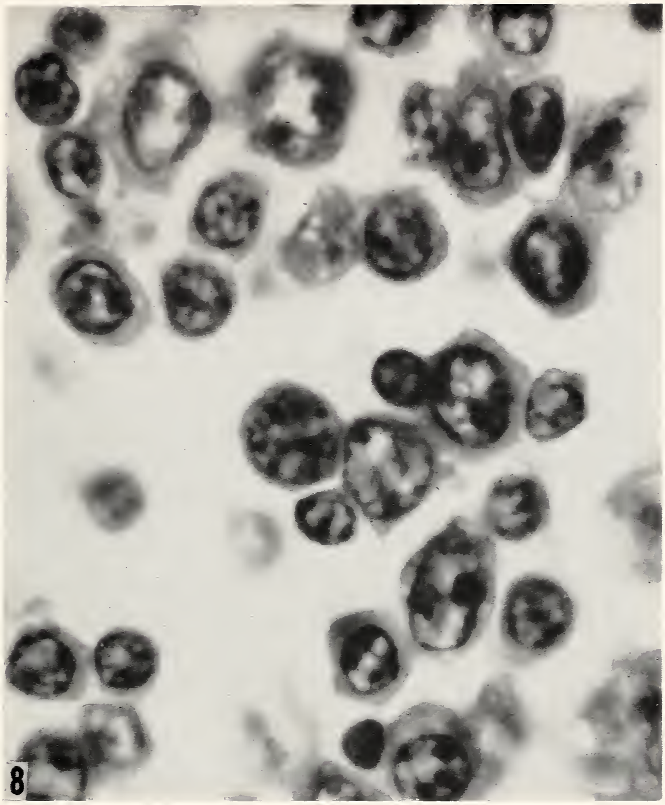

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PLATE 8

FIGURE 9.—Follicular hyperplasia of lymph node from patient with rheumatoid arthritis. Numerous hyperplastic lymphatic follicles extend throughout lymph node, presenting a histologic lesion that mimics follicular lymphoma. Hematoxylin and eosin. × 24 (AFIP Acc. No. 738369, Neg. No. 60-299.)

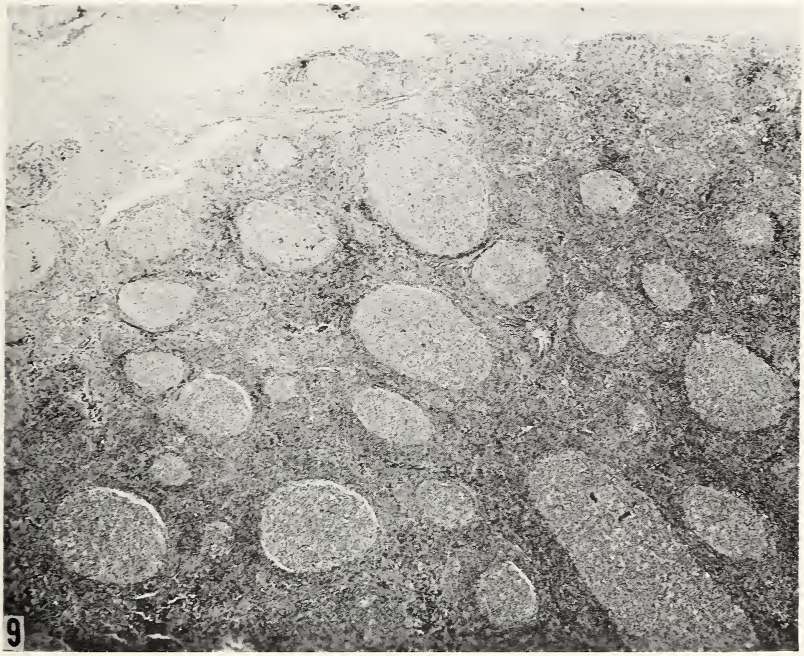
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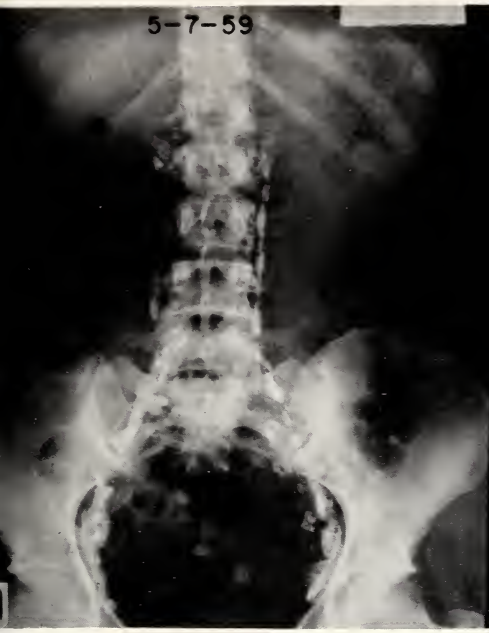
Plate 9

Figure 10.—Ethiodol in retroperitoneal lymph nodes after injection via a lymphatic on the dorsum of the foot.

Figure 11.—Enlarged lymph nodes in patient with chronic lymphocytic leukemia.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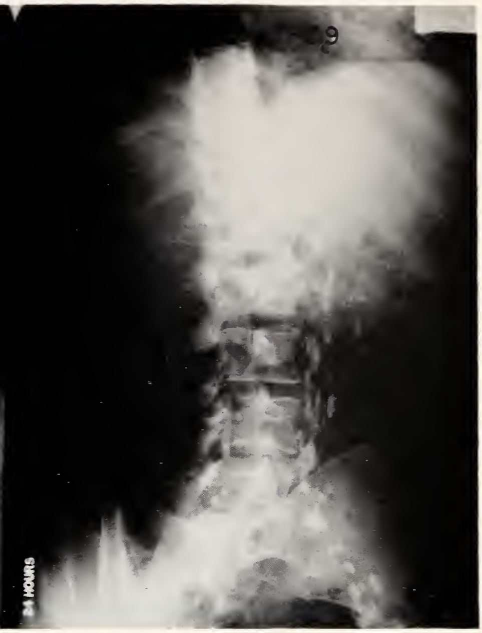
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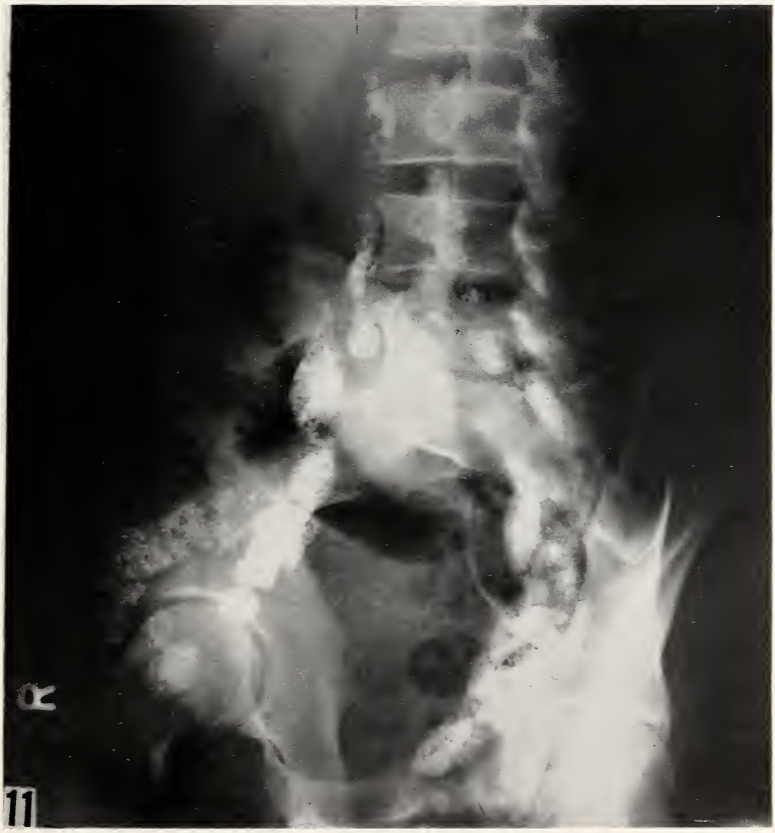
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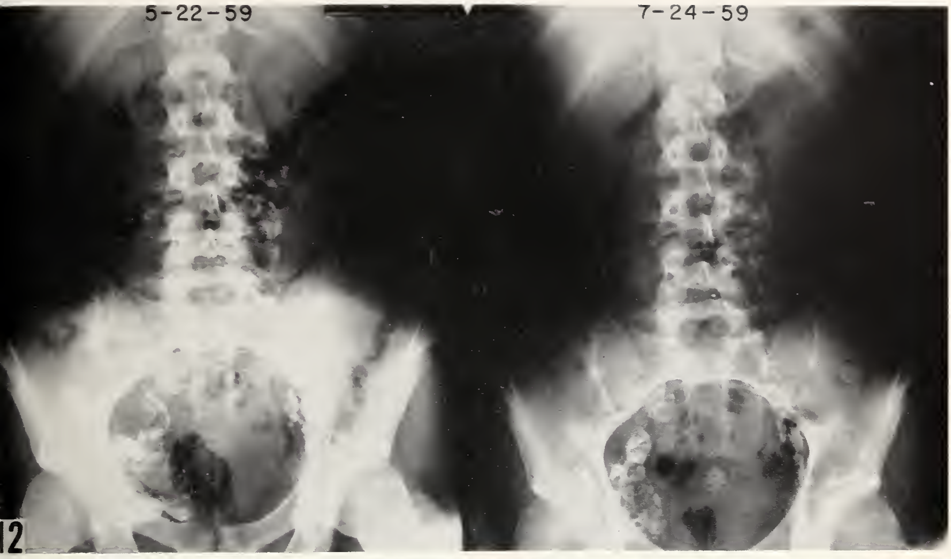
PLATE 10

FIGURE 12.—Large mass of nodes arising deep in pelvis of a 22-year-old male patient with lymphocytic leukemia.

FIGURE 13.—Same patient as in figure 12 after administration of HN2. *Note* decrease in size of nodes.



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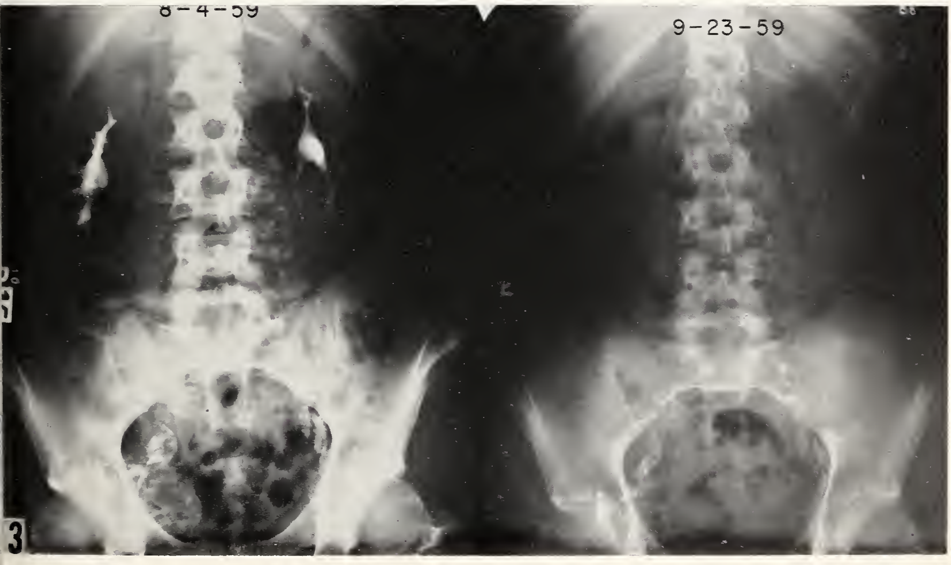

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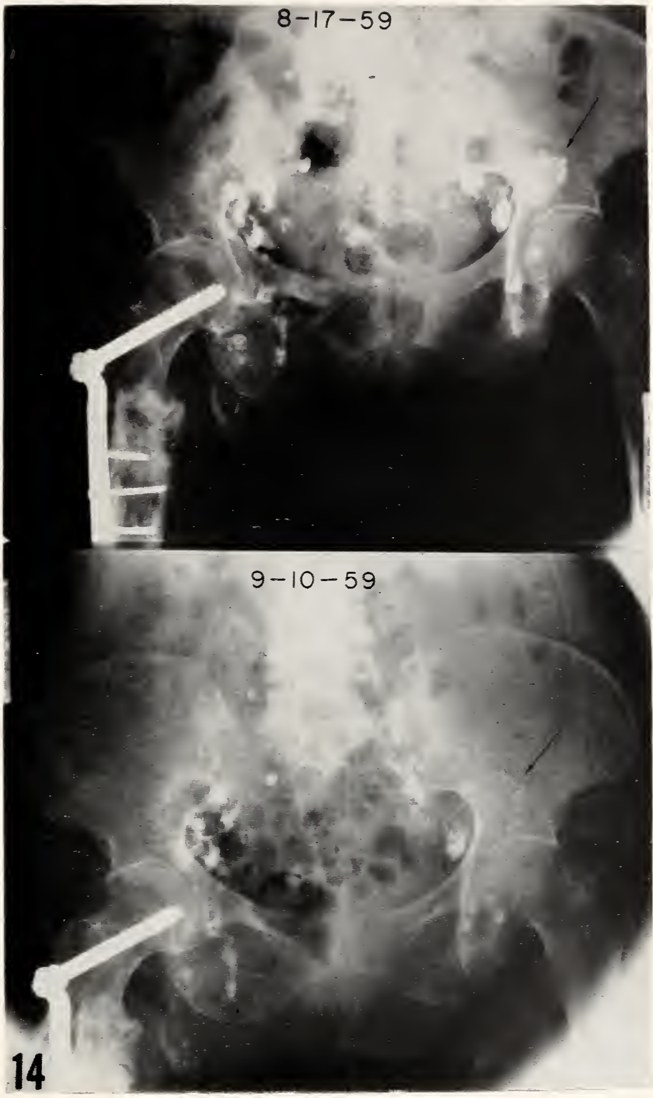
PLATE 11

FIGURE 14.—Increase in mass of lymph nodes, during treatment with methotrexate and dichloromethotrexate, of female patient with lymphosarcoma involving bone area that resulted in pathologic fracture.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14

Therapy of Certain Endocrine and Endocrine-Sensitive Tumors [1]

Chairman: DR. HERBERT BRENDLER, *New York University, New York, New York*

Members of Panel:

Dr. Sidney Werner, *Presbyterian Hospital, New York, New York*
Dr. William Baker, *Massachusetts General Hospital, Boston, Massachusetts*
Dr. Clarence Hodges, *University of Oregon, Portland, Oregon*

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[1] Presented at Conference on Experimental Clinical Cancer Chemotherapy, Washington, D.C., November 11, 1959.

Dr. Brendler: The importance of steroidal or hormonal agents in the treatment of a variety of human cancers is widely acknowledged. The mechanism or mechanisms by which these compounds exert their antitumor effects, however, are comparatively little understood, and this provides another example of the frequent lag of basic scientific explanation for demonstrable clinical fact, which was noted by Dr. Ravdin and others.

In presenting our material, my colleagues and I will endeavor to take up as much of the slack between the two as we can, recognizing the desirability of offering reasonable explanation for these clinical phenomena, if the latter are to prove meaningful in future studies. Much of what we have to report to you admittedly still rests on a somewhat empirical basis, but it is our hope that this discussion and the questions you ask may clarify many points.

As was pointed out by Dr. Baker, the classical boundaries between antimetabolites, alkylating agents, antibiotics, and steroids are gradually dissolving. More and more we are coming to the realization, as in the other three groups of compounds, that the actions of the steroids are dependent in large measure on their interactions with enzyme systems found in normal and malignant tissues. In some instances, hormones have already been shown to act as coenzymes, mediating, for example, conversions of triphosphopyridine nucleotide (TPN) and diphosphopyridine nucleotide (DPN).

Like the other groups of compounds, subtle alterations in the steroid molecule may profoundly affect biological activity of these compounds. Correlations are now being sought between structural configuration and physiological effect of a great many new synthetic steroids. This constitutes, I might say, the prime function of the Hormone Evaluation Committee which is the counterpart, in the steroid area, of the New Agents Committee.

Our subject matter includes tumors involving the thyroid, uterus, adrenal glands, testis, and prostate. The original decision to incorporate these tumors into what seems at first like a somewhat amorphous group was based on the extremely diversified and wide-ranging interests of my predecessor as chairman, the late, much lamented Dr. Delbert Bergenstal—a man whose extraordinary talents and versatility all of us respected deeply and whose loss will be felt for a long time to come. I shall be satisfied if we come close to justifying his high hopes for this panel discussion.

Just as the boundaries between the four major groups of chemotherapeutic compounds are disintegrating with advancing knowledge of mechanisms of action, so we are witnessing within the steroid field itself the gradual dissolution of lines separating androgens, estrogens, progestational agents, corticoids, and anabolic agents. From studies of the intermediary metabolism of steroids, we have learned of conversions *in vivo* of androgens to estrogens and vice versa. In addition, we are discovering that the vast majority of newer synthetic steroids exhibit not one biological property alone but a bewildering array of overlapping ones, which make classification of these agents difficult, if not impossible. Even the older steroids have had to be removed from their comfortable pigeonholes and re-examined in the light of new criteria of biological evaluation.

A number of newer steroids exhibit little or no hormonal activity. There are other compounds, not containing the typical steroid structure, with powerful endocrine properties, of which diethylstilbestrol is a classical example. To complicate the picture even further, it is becoming quite apparent, as with the antibiotics, antimetabolites, and alkylating agents, that tumor-screening studies with steroids are usually of little help in evaluating these compounds for human use. Little if any correlation exists between bioassay data and antitumor data. The indiscriminate use of the results of tumor screening of steroids is fraught with danger unless one can be certain that the tumor used represents a proper yardstick in man. More often than not such information simply cannot be transferred to the clinical level, and the investigator is left with nothing more than an endocrine profile of a particular animal tumor.

I have requested each panel member to present the newer developments in his respective field and to reserve for the question and answer period discussion of more well-known points. We shall attempt to be as factual as possible in presenting our clinical data. In addition, each speaker is privileged to include whatever experimental material seems appropriate. Dr. Sidney Werner will discuss the relationships of treatment of nodular goiter and thyroid cancer.

Dr. Werner: The essential interest of this conference in the thyroid undoubtedly lies in the relation of the gland to the cancer problem as a whole. We are concerned with the role of the thyroid in carcinogenesis and with the effect of cancer on the thyroid. In addition, there has been recent interest in the value of treatment with thyroid hormone or thyroxin in cancer and in the various analogues of thyroxin that have lately been synthesized. Finally, there is the problem of cancer of the thyroid itself and its treatment. Out of these possibilities for discussion, the therapy of cancer of the thyroid was assigned to me, perhaps as an introduction to the broader problems.

It has been thought for many years that there may be a relationship between goiter and the incidence of cancer of the thyroid. Most workers in the field are inclined to believe that the incidence of cancer of the thyroid has not actually increased but rather that more cases are discovered because more patients with thyroid difficulties come to surgery or to clinical investigation. Thus there may be no true causal relationship with iodine deficiency.

On the other hand, people argue that hyperplasia leads to neoplasia. On this theoretical basis, treatment of goiter could decrease the incidence of thyroid cancer. Clinically, however, the peak of incidence for thyroid cancer occurs earlier and is distinct from the peak incidence of nodular goiter. For example, in the very young, the discovery of the nodule in the thyroid is, *ipso facto,* an indication for surgery; the chances are excellent that such a nodule will be a cancer.

In the older age groups, however, the incidence of cancer of the thyroid begins to decline rapidly as the incidence of nodular goiter starts to rise. After age 40 to 60, nodules appear in patients almost physiologically and are only rarely malignant. Of course, with a nodule detected clinically, there is the question of how to tell whether or not it is cancer.

Despite the difficulties with these theoretical considerations, in the last few years there has been a move to try to treat nodular goiter medically. The basic hypothesis is that suppression of the pituitary with thyroid hormone will in turn suppress the thyroid; thyroid cancer will then be prevented by an interruption of the hyperplasia thought to lead to cancer. Also, thyroid of the responsive type, *i.e.*, thyrotropin-dependent, would be successfully treated by the same procedure.

The use of thyroid hormone as a treatment for goiter dates back to the early Chinese and later to the Persians. There have been several subsequent revivals through the years. About 6 years ago, Dr. Astwood and Dr. Greer became responsible for the latest revival of this type of therapy: Their rationale was based on the idea that hyperplasia depends on increased secretion of thyrotropin and that this process may lead to neoplasia.

Assuming that one treats with thyroid hormone a patient who has a goiter or has nodules in the thyroid, one is, of course, attempting to substitute the hormone secreted by the thyroid exogenously. That would then enable the pituitary to be suppressed, the thyroid would not be under constant strain, and the likelihood of conversion to a neoplasm theoretically should be less.

This brings up the questions: (1) Does this method truly prevent cancer formation? (2) If there were a cancer and the cancer were suppressed, would this be definitive or merely suppressive treatment? In 1946 Gardner, using an interstitial-cell tumor of the mouse testis, found that the implanted tumor cells do not grow until estogen is provided. Then a tumor develops. He discovered that such cells can lie dormant for 7 months or more, but still respond to estrogenic stimulation.

Recently, the Fishers, with another tumor, reported in *Science* that these cells, too, lie dormant for long periods, without dying out. In our laboratory, we had a similar experience with experimental mouse thyrotropic tumor originally started by Dr. Furth. For over a year after implantation, no growth of the tumor was seen during thyroxin suppression. Yet when suppression was stopped, tumors appeared shortly thereafter. This suggests that any success with the treatment of cancer of the thyroid in man by thyroid hormone would probably come under the heading of suppressive therapy.

In evaluating the results of prophylactic therapy with thyroid hormone in nontoxic nodular goiter, we must realize, of course, that these are clinical nodules which have not been diagnosed pathologically. Such a series inevitably includes a certain number of patients with chronic thyroiditis, nontoxic diffuse goiter, etc. In any event, about one fifth of patients with nontoxic nodular goiter will show regression of the nodules after treatment. Virtually all the remainder show no such regression. However, Dr. Astwood says that in this group in his own personal series he has not seen any advance in the size of the nodules. Since he has had over 10 years' experience with this method, and since it is well known that the incidence of cancer in nodular goiter is high, in some figures as high as 20 percent, he must be treating cancer and possibly suppressing it. However, one must remember the remarkably slow course of this disease.

In contrast, at the Presbyterian Hospital in New York, we have in our series similarly treated at least 5 patients in which nodules increased in size. Of these, 3 had surgery and the nodules were cancerous. The other 2 were known already to be cancerous, but increased in size with therapy.

The questions of how to appraise any therapy of thyroid cancer are many. First, what is cancer of the thyroid? There is a histologic classification, groups 1, 2, and 3, meaning from mild virulence to very severe virulence. Yet the histologists are the first to say they cannot always be sure.

Second, there is the dose level for thyroid-hormone administration. Dr. Thomas has pointed out that increases in the dose of thyroid hormone to levels which will raise the metabolic rate considerably may then induce responses of the cancer when there was no previous response.

Finally, there is the prolonged spontaneous course of the disease in some patients. An example is that of a young woman first seen in 1954 with cancer of the thyroid of the papillary type. She had cervical-node involvement and had had a series of operations at another institution. There was every evidence that the disease was progressing. In 1956 it was clear that she had pulmonary metastases. At that time she was first put on tri-iodothyronine, 100 μg. daily.

In 1957 there was not much change. However, by that time the cervical-node enlargment had completely disappeared. In 1958 there was a definite increase in the size of the lung infiltrate, though symptomatically the patient was well.

By 1959 a tissue mass reappeared in the region of the right lobe of the thyroid, but with increased doses of tri-iodothyronine there was some evidence that perhaps this increase had been not only arrested but reversed.

Another patient with obvious advanced mitotic lung lesions showed only very slow advance and no specific deterioration from 1938 through 1954. Thus the appraisal of any treatment of thyroid cancer with thyroid hormone or thyroid analogues is open to question because of the variable and slow spontaneous course. Much further investigation is required.

Dr. Brendler: Dr. William Baker will discuss the treatment of endometrial carcinoma with progestational agents.

Dr. Baker:[2] The successful treatment of cancer of the uterus is usually accomplished by total hysterectomy, irradiation, or a combination of both (*1*).

Data from 27 clinics, in 8,366 cases, are surveyed in the *Tenth Annual Report of the Results in Treatment of Carcinoma of the Uterus* (*2*). The over-all 5-year cure rate was 54.3 percent. When the tumor was confined to the uterus (Stage I, Group I), the 5-year cure rate was 60 percent, but was only 19.6 percent for those patients in whom the growth had spread outside the uterus (Stage II) (*2*).

Metastatic inoperable carcinoma of the uterus is usually treated by irradiation, and much symptomatic benefit can be achieved by this form of therapy. Recurrent local disease after radiotherapy has been a problem in management. Also, the treatment of lung metastases is less successfully managed by radiotherapy, since adequate radiation to metastatic lung lesions carries with it the hazard of producing pulmonary fibrosis.

The beneficial effects of changes in the hormonal environment of certain metastatic tumors of the prostate and breast prompted us to investigate the use of sex hormones in the treatment of endometrial cancer. The relationship of sex hormones to human endometrial cancer is not well defined at present. The coexistence of endometrial hyperplasia and endometrial carcinoma in humans has given rise to the concept that endometrial cancer may be related to overstimulation with estrogens (*3*). Endometrial cancer has been induced with long-term estrogen administration in guinea pigs and rabbits (*4*), but long-term administration of progesterone in rabbits has not as yet produced endometrial cancer (*5*).

Progesterone has a profound effect upon the endometrium and, synergistically with estrogen, produces the secretory endometrial phase of the menstrual cycle (*7*). If progesterone is continued alone over a prolonged time, disappearance of acini will occur with marked decidual reaction of the endometrium (*7, 8*). This latter effect is usually obtained without any consistent measurable effect upon the urinary gonadotropins (*7*). Because of the known effects of progesterone on the uterine endometrium, we felt that this steroid was worthy of trial in the treatment of metastatic endometrial carcinoma, which could not be effectively treated by radiotherapy.

In 1951 we began treating patients who had metastatic recurrent endometrial carcinoma with progesterone or progesterone-like compounds. At the present time, we have treated 15 such patients and it is the purpose of this report to depict the results obtained. All 15 patients we have treated had a previous pathologic diagnosis of adenocarcinoma of the endometrium. Most of them had been previously treated by panhysterectomy, local radiotherapy, or both treatments in conjunction. No patients are included who had not been treated for longer than 3½ weeks on progesterone therapy. Five with metastatic adenocarcinoma of the uterus have shown objective regression of metastatic disease, lasting from 6 months to 4½ years. In 4 of these 5, the metastatic disease was evidenced by lung metastases and thus no biopsy material was available. In the fifth patient, local disease was present, but biopsy material was not obtained. In all patients that have responded, gratifying symptomatic relief was obtained with objective remission of the metastatic chest lesions or local disease. At the present time, 2 of the 5 responders have died of recurrent disease and the 3 living patients now have evidence of recurrent disease, either locally or within the chest.

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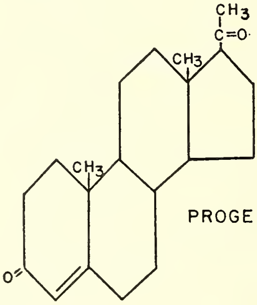
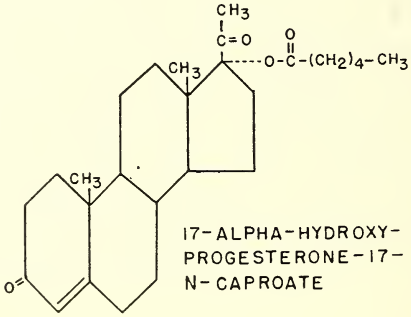
[2] In collaboration with Dr. Rita M. Kelley.

Early in our studies we administered progesterone intramuscularly in the form of Proluton [3] at a dose level of 50 to 200 mg. daily. More recently we have been using Delalutin [4] in a dose schedule of 250 to 500 mg. twice a week. The structural formulas are shown. The 5 cases that responded are reported in detail, and the 10 patients not responding are summarized in table 1.



PROGESTERONE



17-ALPHA-HYDROXY-PROGESTERONE-17-N-CAPROATE

Case 1: A.G., MGH 572590, age 61.—In 1945 a panhysterectomy was performed for adenocarcinoma of the endometrium. In 1947, a recurrence in the vagina was treated with 4200 r with a good response. In October, 1950, the patient developed a cough, anorexia, and lost 24 pounds over the preceding 3 months. She was seen by us in November, 1950, when a chest X ray was taken (fig. 1). On November 14, 1950, the patient was given 200 mg. of Proluton intramuscularly daily (4 cc.). Follicle-stimulating hormone (FSH) titer taken before therapy was positive for 52 mouse units and negative for 104 mouse units. Two weeks after beginning Proluton therapy, she felt symptomatically much improved. Repeat chest X ray at that time showed no change. One month after start of therapy, she continued to improve with a gain in weight of 5 pounds, and again no change was seen in her X rays. On January 14, 1951, improvement was maximal with no cough, and X rays showed definite diminution of all lung metastases. On March 22, 1951, further X rays showed more regression in the right upper-lobe lesion (fig. 2). The patient was continued on Proluton, 200 mg. daily, and no further regression by X ray was obtained in the chest. In June, 1951, since her condition was static, she was put on Proluton, 100 mg. 3 times a week, and in September, 1951, definite increase in the size of the right upper-lobe lesion and left upper-lobe lesion occurred. In October, 1951, there was further increase in the size of the lesions, and in November, 1951, X-ray therapy was given to the right and left upper lobes, 1800 r to each area. X-ray therapy was completed on November 23, 1951. The patient became progressively worse and died in December, 1951. Permission for autopsy was refused. Total dose of Proluton was 38,400 mg.

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[3] Progesterone in oil. Schering Corp., Bloomfield, N.J.

[4] 17-α-Hydroxyprogesterone-17-*n*-caproate. E. R. Squibb & Sons, New York, N.Y.

Case 2: R.W., MGH 845077, age 61.—In March, 1951, a panhysterectomy was done for adenocarcinoma of the uterus with metastasis to one ovary. Local vaginal recurrence in April, 1954, was treated by 3300 r. One month after completion of X-ray therapy, she had multiple nodular densities in both lungs, and was referred to our clinic. At that time, metastatic series was negative. Serum calcium was 12.5 mg. percent, phosphorus was 2.3 mg. percent, and alkaline phosphatase was 3.3 Bodansky units. In June, 1954, a repeat X ray of her lung indicated increase in the nodular densities in both lung fields (fig. 3). She was placed on Proluton, 50 mg. 3 times a week, intramuscularly. In September, 1954, repeat X rays showed definite regression of her chest metastatic lesions (fig. 4), and in 1 year all the nodular densities had disappeared (fig. 5). Because of this dramatic response, she was continued on Proluton, 50 mg. 3 times a week for the next 4 years, and no change in chest X rays occurred during periodic examinations. She tolerated the medication well. In March, 1958, a small increase in the right hilum of the chest was seen and, over the next 6 months, gradually increased in size. In September, 1958, because of increase in size of the hilar mass (fig. 6) Proluton was discontinued, to see whether a withdrawal response could be obtained. No change occurred after discontinuance and over the next 10 months no therapy was given. In July, 1959, further increase in hilar disease was seen in her chest, and Delalutin therapy, 500 mg. twice a week, was begun. X rays taken in December, 1959, showed definite but slight decrease in size of the hilar metastatic disease, and a peripheral nodular lesion of the right lower-lung and left upper-lung fields had disappeared. She is at present alive, well, and being continued on Delalutin 500 mg. twice a week. Hypercalcemia and hyperphosphatemia have persisted throughout this entire period. Metastatic series including bone biopsy are negative for metastatic disease. She has refused parathyroid exploration. Total dose is 26,000 mg. first course and 20,000 mg. second course, to date.

Case 3: R.H., MGH 961541, age 64.—In February, 1957, a dilation and curettement was performed because of intermittent vaginal spotting. A diagnosis of adenocarcinoma of the fundus was made. Ether examination revealed implants in the vulva and enlargement of the uterus to 3 times normal with penetration of posterior uterine wall. X rays of her chest revealed bilateral nodular densities with increased linear markings in both lung fields, interpreted as lymphatic spread of carcinoma. Primary disease in the uterus was treated with 5400 mg. hours of radium. Because of the metastatic disease in her chest, Delalutin therapy, 250 mg. 3 times a week, was begun. She was treated with this compound from March, 1957, to May, 1957. In 1 month her chest X ray had begun to clear and in 2 months it was completely negative. All palpable local disease disappeared, but vaginal smears were positive intermittently. Chest X rays have remained free of disease, but, in August, 1959, retroperitoneal recurrence appeared. At the present time she is on her second course of Delalutin therapy, 250 mg. 3 times a week, but is getting no response to this second course and her condition is gradually declining. X rays of her chest were lost and are not available for reproduction.

Table 1.—Summary of 10 patients not responding to Proluton and Delalutin

Patient, unit No., age	Prior treatment	Extent of disease	Duration of treatment and total dose	Response
E.R. PH 40789 59	8/56–H* + 5000 r; 6/57–1800 r	8/57–Huge mass in vagina; invasion of bladder; multiple nodules in chest	Proluton, thrice weekly 100 mg. 36 weeks, 12,000 mg.	No change in pulmonary disease; pelvic exenteration 8/58 because of progression of symptoms; died 12/58 at home; no postmortem
R.H. MGH 736216 55	4/51–R† 4400 mg. hrs.; 6/51–5000 r	11/51–Supraclavicular Horner's on left; pulmonary and pelvic wall metastases	Proluton, 200 mg. biweekly 4 weeks 200 mg. thrice weekly 1 week, 5400 mg.	Progression to death 1/6/52; massive peripheral edema
B.K. MGH 1041561 51	5/58–H + 4000 r	1/59–Mass in left supraclavicular area; mass in pelvis; bilateral leg edema	Delalutin, 500 mg. biweekly 5 weeks, 5000 mg.	Two weeks: regression of neck and abdominal masses, then progression to death in 5 weeks
C.P. MGH 795784 33	1/52–H; 1/53–1800 r	1/53–Local recurrence; extensive pulmonary metastases	Proluton, 200 mg. biweekly 4 weeks, 100 mg. biweekly 8 weeks, 7200 mg.	No response; steady decline; died 5/53 at home; developed acne
N.S. MGH 668882 47	6/49–H; 3/50–5000 r	2/51–Massive right pleural effusion positive for tumor cells; multiple pulmonary metastases	Proluton, 200 mg. once daily 6 weeks, 100 mg. once daily 1 week, 8000 mg.	No response; repeated thoracenteses required; died 5/3/51 (buttock abscess)
F.E. MGH 729814 73	3/51–H + 1800 r (no chest film)	5/51–Local recurrence; multiple pulmonary metastases and metastases in pelvic bones	Proluton, 200 mg. once daily 4 weeks, 5600 mg.	No response; died at home 7/7/51
M.H. MGH 860451 63	1949–H + X ray (? amount); 2/55–4000 r	3/57–Mass in left pelvis; partial intestinal obstruction	Delalutin, 250 mg. biweekly 8 weeks, 4000 mg.	No response; died in terminal-care hospital

I.P. MGH 836647 62	6/52–H; 10/53–9600 r (skin dose)	2/54–Vaginal recurrence right lower abdomen and pelvic wall; involvement of iliac vessels with lymph obstruction right leg	Proluton, 200 mg. once daily 2 weeks, 100 mg. once daily 4 weeks, 5600 mg.	No response; developed pleural effusion; died shortly after discharge
S.A. PH 39634 64	1947–H; 1954–colostomy; 11/56–large bowel obstruction	12/56–Huge right lower quadrant mass and many smaller tumor masses; bilateral leg edema; terminal state	Delalutin, 100 mg. once daily 2 weeks, 250 mg. biweekly 12 weeks, 11,200 mg.	Marked subjective improvement for 3 months; appetite stimulation, loss of edema, resumption of activity; partial obstruction 4/57; died at home 8/58
R.W. PH 51152 58	4/57–R 300 mg. hrs. ext. radiation (? amount)	11/57–Recurrence; metastases to vagina, vulva, perineum, skin; phlebitis and edema right leg; in terminal state	Delalutin, 250 mg. biweekly 3 weeks, 1500 mg.	No response; died 12/19/57

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*H = Panhysterectomy.

†R = Local radiation or radium.

Case 4: V.C., PH 39432, age 55.—In March, 1951, a panhysterectomy was performed for adenocarcinoma of the endometrium. Postoperative irradiation of unknown amount was given at the operative site. In March, 1956, because of loss of weight, lymphedema of the left leg, recurrent partial intestinal obstruction, and a mass felt on physical examination, abdominal exploration was done and a mass was found fixed to the left pelvic wall extending up into the left upper quadrant. Intravenous pyelogram examination showed that the left kidney was not functioning. Multiple pulmonary metastases were seen, 1 to 3 cm. in diameter, and in November, 1956, because of metastatic pulmonary disease, Delalutin 250 mg., 3 times a week, was begun (fig. 7). This was continued until March, 1957, when the dose was reduced to 250 mg. twice weekly until December, 1958. In March, 1957, there was definite decrease in metastatic pulmonary disease, disappearance of lymphedema, and marked improvement in bowel function. The left lower-quadrant mass was no longer palpable and the left kidney still did not function. In February and November, 1958, X rays revealed continued decrease in pulmonary metastases (fig. 8). In December, 1958, she developed intestinal obstruction and was treated at an outside hospital. She died at home in February, 1959. Permission for autopsy was refused.

Case 5: M.J., MGH 1009968, age 67.—In March, 1957, a vaginal hysterectomy was performed for adenocarcinoma of the endometrium. This was followed by 3000 r of X-ray therapy. One year later there was local recurrence in the vagina with multiple ulcerating nodules in the anterior and posterior walls of the vagina. The local disease disappeared with 2000 mg. hours of radium. In December, 1958, new nodules appeared in the vagina, multiple in number, measuring from 1 to 4 cm. They were ulcerated and bleeding, and Delalutin therapy was begun, 500 mg. twice a week, and continued until March, 1959, when the nodules showed definite evidence of decrease in size, and 250 mg. of Delalutin was given twice a week. In June, 1959, all evidence of ulcerating nodules had disappeared and she was begun on 375 mg. of Delalutin weekly. In September, 1959, she developed steady pain in the left lower quadrant, palpable disease in the vaginal apex, and a barium enema revealed an extrinsic mass elevating the rectosigmoid junction. Delalutin was discontinued and she received 2000 r of X ray to the left lower quadrant with relief of pain. She now has advancing disease throughout the pelvis and is slowly declining.

These 5 patients represent definite objective regression of metastatic endometrial adenocarcinoma after progesterone therapy. How progesterone affects metastatic endometrial carcinoma is unknown. The major action is probably one of direct local effect on the endometrial carcinoma in much the same way that progesterone affects normal endometrial tissue. It is of interest that approximately one third of the patients treated responded objectively to administration of progesterone. This is approximately the same percentage that responds to other forms of hormonal management in carcinoma of the breast. These tumors probably represent slowly growing tumors, which might be deemed hormonally dependent carcinomas. In none of the patients have we found any indirect evidence of estrogen activity as measured by changes in vaginal smears or by a measurable increase in estrogen excretion performed in one patient. There also has been no evidence of androgenic activity, as measured by hirsutism, acne, increased libido, or increased clitorial size. Because of the large doses of Delalutin which we have used, we have done repeated and frequent measurements of pH and CO_2 on 2 of the 5 patients and in neither of which have we found a lowering of the CO_2 or change of the pH to the alkaline side. An FSH titer done on 1 patient, R.W., during therapy was 80 rat units, indicating no suppression of FSH. No luteinizing hormone measurements have been performed. Two patients showed evidence of peripheral edema with weight gain and ankle edema, beginning approximately 2 weeks after therapy was instituted. In 1 patient who did not respond, massive edema developed which was thought to be due to the salt-retaining effect of progesterone. In no case, after discontinuance of progesterone, have we noted a change in the vaginal smear or withdrawal bleeding. Since progesterone has a key role in steroid metabolism, it is possible that progesterone could be acting through one of its many metabolic end products: estrogens, androgens, or corticoids. As stated before, we have no evidence that appreciable conversion to estrogen or biologically active androgen has occurred. Aldosterone and corticosterone levels were not obtained.

To summarize: Fifteen patients with metastatic adenocarcinoma originating in the endometrium have been treated from 3½ weeks to 4½ years with progesterone in varying quantities, from 50 mg. 3 times a week in the form of Proluton to as much as 1 gm. of Delalutin weekly. Five out of 15 cases showed definite objective regression lasting from 6 months to 4½ years. In none of the patients do we have available pathologic material to document the objective benefit obtained with the administration of the drug, nor have we noted any evidence of estrogen effect as measured by vaginal-smear change or pigmentation of nipples or withdrawal bleeding. Progesterone seems to be an effective compound in the treatment of some metastatic endometrial adenocarcinoma and should be given a clinical trial in all metastatic endometrial carcinoma that is not available to other forms of therapy. Because of the small number of patients treated in this series, the exact percentage responding to progesterone is unknown. It is of interest that the percentage obtained in this small series corresponds to the approximate percentage of patients who usually respond to hormone therapy in breast carcinoma. The exact mode of action of progesterone in endometrial carcinoma is unknown, but the major effect is undoubtedly related to its direct local effect, which occurs on the endometrium in the absence of endometrial carcinoma. We believe this is another example of a target-organ cancer that retains its hormonal identity and responsiveness in spite of neoplastic transformation.

Dr. Hodges: Adrenal carcinoma has extremely interesting endocrine, as well as ordinary, tumor characteristics. We and others found a number of years ago that corticosteroids failed to influence adrenocortical carcinoma and that this failure served as a test for differentiating adrenocortical hyperplasia from adrenocortical carcinoma.

There was little that was encouraging in the line of chemotherapy until Dr. Hertz's work, reported in 1956, in which he found suppression of adrenocortical carcinoma but also extreme toxicity with the use of the compound, amphenone. Subsequent studies with related compounds gave rise to the work by the late Dr. Delbert Bergenstal, presented by Dr. Hertz at the

Vergennes' meeting in September, 1959. I should like to review this work because of our great interest in this particular field.

Dr. Bergenstal's series included 13 patients, all of whom had adrenocortical carcinoma. Twelve of these had hormonally functioning tumors with increased plasma and urinary levels of 17-ketosteroids and 17-hydroxycorticosteroids; 1 was a nonfunctional tumor. The age range was from 7 to 57 years. Cushing's syndrome was present in 7 and virilization in 11 patients. There were metastases to the lungs and to the intra-abdominal organs.

The compound used was ortho, para prime isomer of diphenyldichloroethane (DDD), an analogue of DDT. The dose was 1 gm. per day initially with gradual increase up to 10 gm. per day over a 2-week period, though some doses ran as high as 15 gm. per day. Because of the adrenocortical insufficiency that developed, Meticorten or Decadron was used for maintenance therapy. Toxicity included anorexia, vomiting, skin rash, mental depression, diarrhea, and tremors.

Use of this compound caused disappearance of pulmonary metastases and regression of the primary tumor. Sustained treatment was necessary; however, in some instances, temporary lapses of treatment were well tolerated, apparently because of storage of this material within body fat. There were 6 patients who showed remissions up to 18 months. 17-Ketosteroids and 17-hydroxycorticosteroids were significantly reduced in 11 out of 12 patients. Interruption of therapy in one instance allowed a clinical exacerbation.

Dr. Monte Greer of our endocrinology department has used ortho, para prime DDD on a patient with adrenocortical carcinoma which was nonfunctional; no effect was noted.

We may turn then to a consideration of neuroblastoma, the highly malignant, swiftly metastasizing tumor of the adrenal medulla, which we see particularly in children under 3 years of age. We know that the history of this tumor is generally a rapid progression, with bone and liver metastases, and exitus usually within 2 years. There are rather striking departures from this general pattern. Occasionally the tumor will disappear after a biopsy. Spontaneous and complete regressions have been recorded. Occasionally, they will regress for periods after surgical exploration, radiation, and other rather nonrelated types of therapy.

A series reported by Tan, Murphy, Dargeon, and Burchenal, with actinomycin D, an antibiotic, showed 3 out of 6 patients who had subjective remission, and 1 of these showed an objective decrease in size of scalp nodules. Dr. Gross has recently emphasized a significant principle in this type of therapy (and this is pleasing to me, as a surgeon, that the field has not entirely been lost); if one can remove as much of the tumor mass as possible, the chances for improvement with X-ray and antibiotic therapy are greatly increased.

In the series reported by Gross, he does not state which of the chemotherapeutic agents was employed, but perhaps Dr. Sidney Farber will tell us this later. We have seen a rather interesting regression for 1 year in a 3-year-old boy with disseminated neuroblastoma, treated with testosterone alone.

We may turn now from the adrenal to the testicular carcinoma. Information on this carcinoma is scanty, the series are small, and the remissions are somewhat less than optimal, but most urologists have agreed on these classifications: (1) seminoma, (2) embryonal carcinoma, (3) teratocarcinoma, with either seminoma or embryonal carcinoma component, and (4) adult teratoma.

I want to call your attention to the principle elaborated by Farber, which is exemplified in the case history of a 47-year-old man with a painless, slightly enlarged, hard testis. Orchiectomy revealed a teratocarcinoma with embryonal carcinoma component. Four pulmonary metastases were present. Teratocarcinoma presages an unhappy fate as a rule: usually 80 percent of its victims are dead within 2 years. Surgery, by resection of lymph-node chains, adds slightly to survival; X-ray therapy is not too effective. In this patient, on X-ray therapy alone, the pulmonary metastases were multiplying. At this point, and at the suggestion of Dr. Farber, actinomycin D was administered and then further X-ray therapy was given. There was a rather prompt disappearance of 4 pulmonary nodules and a remission that has been sustained through the 13 months up to the present time. Farber has postulated that tumor cells may be sensitized to irradiation by the chemotherapeutic agent.

The largest series has been that reported by Blochin in Moscow. I shall call this compound phenylalanine mustard rather than Sarcolysin. Of 62 patients, 27 had seminoma; 25 of these were benefited. Of 12 patients with choriocarcinoma, only 1 showed tumor regression.

In an attempt to find better methods of chemotherapy, the group at New York Memorial Center for Cancer and Allied Diseases has used combined cancer chemotherapy. This regimen includes an antibiotic (actinomycin B), an antimetabolite (methotrexate), and an alkalating agent (chlorambucil). This regimen is employed over a 10-day period, followed by a 10-day rest period, a second treatment of 10 days, etc. In a series of 10 patients with testicular tumors, 2 showed excellent remissions, 3 showed partial regression, 2 showed slight effect, and 1 showed no effect. The best results were obtained with embryonal carcinoma. In a personal communication, Whitmore states that the response rate in a series of over 20 testicular tumors is now slightly over 50 percent.

With regard to testicular choriocarcinoma, Li, Whitmore, and Golbey reported at the American Association for Cancer Research meeting in April, 1959, that of 9 patients with this type of tumor, and all showing high urinary chorionic gonadotropins, 2 showed a return to normal of high gonadotropin titers and striking regression of pulmonary metastases, lasting 1 to 7 months.

At our institution, of 8 patients who received nitrogen mustard for disseminated testicular cancer, 2 with embryonal carcinoma showed good response. Four patients with seminoma, 1 with embryonal carcinoma, and 1 with teratocarcinoma showed no response. Estrogens (diethylstilbesterol) had no effect in 5 patients. Androgens (testosterone) had no effect in 4 patients. Contralateral orchiectomy was ineffective in 1 patient.

In general, with testicular cancer, remissions induced by chemotherapy are definite in 50 percent of patients but tend to be incomplete and transient. Embryonal carcinoma and seminoma show the most marked response. Avenues of investigation tend toward (*a*) combinations of chemotherapeutic agents, and (*b*) higher doses.

Dr. Brendler: Our last subject is prostatic cancer and I will attempt to cover this as concisely as possible so as to leave some time for questions. Prostatic cancer, as you know, is an extremely common disease in men over the age of 50, probably the commonest form of malignancy in the aging male.

For the last 20 years the initial therapy of patients with advanced, disseminated prostatic cancer has been characterized by the use of methods designed to nullify the effects of endogenous androgens; castration and estrogen administration have been standard approaches. With these techniques, a favorable response has been obtained in 60 to 80 percent of the patients treated. Nevertheless, after varying periods of remission, reactivation has occurred. The subsequent treatment of relapse has largely been directed at the elimination of extra-gonadal androgens by cortisone administration, adrenalectomy, intensified estrogen therapy, and even hypophysectomy.

The treatment of such reactivated cases has generally proved disappointing, though from time to time there have been some beneficial responses recorded. During the past 14 or 15 months, the cooperative study in prostatic cancer has attempted the clarification of some of the problems associated with relapse in this disease. The aim at the present time is to study prostatic cancer in patients who have either proved refractory to initial therapy or who have relapsed after a previously beneficial response. Previously untreated cases are not included because, since about 60 to 80 percent respond satisfactorily in the beginning, it would be an enormous undertaking to attempt to find an agent which would improve on the classical regimen.

At the present time, there are 12 medical centers cooperating in this study. Since the earliest planning stages of the study, the statistical center, which is located at New York University, has participated closely in all phases of the cooperative program, including protocol design, random allocation of patients at all participating institutions, constant surveillance of data, and statistical evaluation of results on a continuing basis.

The pathology center, located at Columbia University, Frances Delafield Hospital, reviews histological slides on all potential study patients to confirm the tissue diagnosis. No patient may be admitted to the study without such tissue confirmation.

At present, each clinical trial is designed as a double-blind comparison of the effectiveness of two different therapies. Each trial is planned so that it may be carried out simultaneously at all participating institutions. This method of simultaneous testing has been adopted by the study group, at least for the present, to assure ample case material for statistical analysis.

The first 3 trials, which are currently in progress, consist of (1) an evaluation of diethylstilbestrol at 5 mg. per day versus a placebo given orally; (2) diethylstilbestrol, 5 mg. per day versus diethylstilbestrol 500, also orally; (3) testosterone propionate, 300 mg. per week versus diethylstilbestrol, 30 mg. per week.

Subsequent trials now being planned involve the use of potent corticoid and progestational agents, as well as a number of the newer steroids manifesting a variety of biological properties in animal studies.

To date, 122 patients have been admitted to the first trial. Of these, 99 have completed the trial, and 66 have been admitted to the second trial, of whom 58 have completed it. Each of these trials requires a fixed sample of 200 patients, 100 tests, and 100 controls. Twenty-three patients have been admitted to the third trial; of these 18 have completed the trial. This trial is being evaluated by a sequential procedure so that the number of patients required is not fixed.

Now, in view of the double-blind nature of these trials, the results will not be made known by the statistical center until each trial has been completed. Nevertheless, it is possible to record at this time a number of clinical impressions which have developed.

On the first trial, diethylstilbestrol versus placebo, which is well past the half-way mark, experiences with patients, in whom the code has been broken for one reason or another, convey the distinct impression that diethylstilbestrol is slightly, but definitely, more effective than placebo in the treatment of relapse. It is only fair, however, to point out that a few objective remissions have actually been observed in patients receiving placebo. I think that what I have just said makes it worthwhile to repeat the admonition of our presiding chairman, Dr. Shimkin, at a recent meeting of the Clinical Studies Panel, to the effect that most so-called clinical responses are in reality *responses by definition.* It simply depends on how the protocol is worded and what conditions are set down as criteria of therapeutic effect which determine these relative cures. We must not forget that what we are really looking for are methods which will totally abolish the cancer, or at least if not abolish the cancer, make the patient more comfortable and prolong his life.

I want to say a few words about new compounds. The prostate study group has a subcommittee that is continually investigating at both experimental and clinical levels the many new synthetic compounds, mostly steroids, coming our way. All the members in the group are encouraged to carry out pilot trials on patients who do not qualify for the formal protocol trials. At the present time, no new compounds have been found in the clinical pilot trials that are consistently effective in the treatment of reactivated prostatic cancer. We see objective remissions sometimes, but subjective improvement occurs more often and then presumably on an anabolic basis.

The use of alkylating agents, antimetabolites, and antibiotics has been reported sporadically during the last few years. Although our study group is concentrating on steroids at present, a number of its members are carrying out pilot studies with some of these agents. Should a promising compound be discovered as a result of a pilot study, a formal trial of such a compound would certainly be included in the cooperative program.[5]

* * *

We are now ready for questions or comments. I have a question for Dr. Werner: What is the status of androgen in thyroid carcinoma, how effective is it, what would be the rationale, if any, for its use in this disease?

Dr. Werner: There are to my knowledge 3 excellent results with testosterone propionate given to cancer of the thyroid of the papillary type. The rationale is the well-known increased incidence of all thyroid disease in women, which also holds in thyroid cancer, the ratio being something like 3 women to 1 man.

Whether a sex difference can be attributed to a hormone or not is problematical in view of the genetic sex determination, as well as the actual secretion of hormones, but in this instance androgens have been successful. However, they have not been tried in a broad spectrum of disease and this remains to be done.

Dr. Brendler: Dr. Baker has a comment that he would like to read.

Dr. Baker: "I would like to add that two patients with endometrial carcinoma recurrence, treated with Delalutin at the Free Hospital for Women, had objective evidence of remission." I am glad that somebody else is getting objective evidence of remission with progesterone.

Dr. Brendler: A question for Dr. Baker: Are the progesterone-like drugs effective against cervical carcinomas?

Dr. Baker: This question prompted two papers, one originally depicting the effectiveness of progesterone in carcinoma of the cervix and the second a retraction of the data. One can say that progesterone causes no objective evidence of regression of cervical carcinoma. It does seem to clean up the cervical mucus and it also does seem to make the cervical cancer look a little better, but it does not seem to have any other effect on it.

Dr. Brendler: The question is asked: What is the outlook for zinc compound therapy in cancer of the prostate? I am not sure precisely what that means, unless the question refers to the use of zinc chelating compounds, like diphenylthiocarbazone, which is known to bind zinc. Zinc is an important constituent of the normal prostate as well as the malignant one. It is tempting to think that an agent of this sort might be valuable. A number of patients actually have been treated with this compound at the Memorial Center in New York City. So far as I know, there have been no dramatic results. It is a fairly toxic compound and I think its use must be undertaken with caution. However, I do think this is an approach that should be looked into.

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[5] Part of this material was presented at the Conference on the Biological Activities of Steroids in Relation to Cancer, sponsored by the Cancer Chemotherapy National Service Center, Vergennes, Vt., Sept. 27 to Oct. 2, 1959. Academic Press, Inc. In press.

The last question, Should diethylstilbestrol be given to patients who have been castrated for prostatic cancer? This is a difficult question to answer at this time. Indeed, it constitutes the *raison d'être* of our first and second cooperative trials. Pending the outcome of our study, however, I would advise every patient with advanced prostatic cancer, whether castrated or not, to begin diethylstilbestrol therapy promptly. The dose could be anywhere between 5 to 25 mg. daily.

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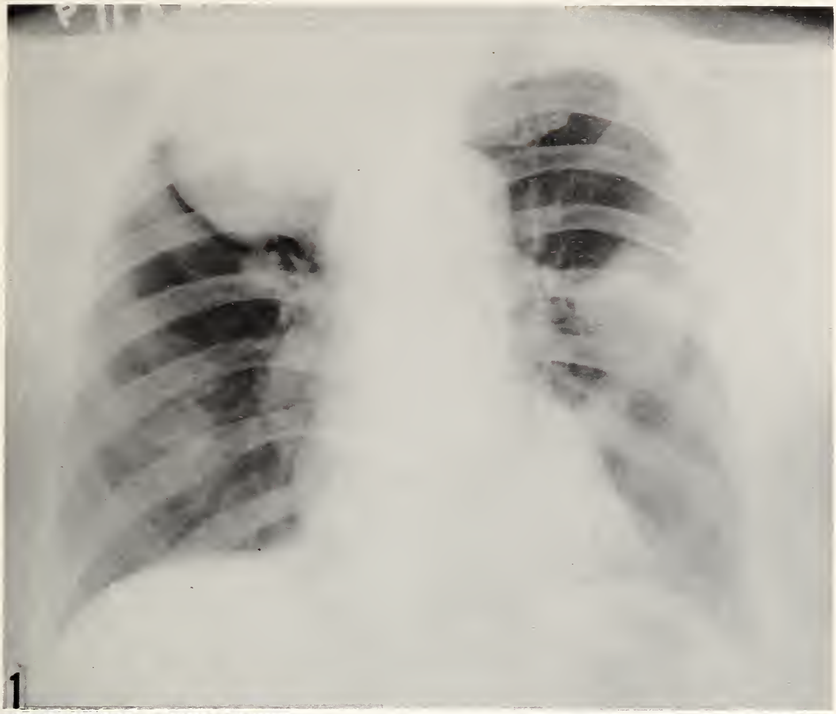
PLATE 12

FIGURE 1.—A.G., MGH 572590, 11/2/50. Multiple pulmonary metastases.

FIGURE 2.—A.G., MGH 572590, 3/22/51. *Note* disappearance of lesions in *right* midlung field and marked shrinkage of lesions in *right* upper lobe and *left* midlung.



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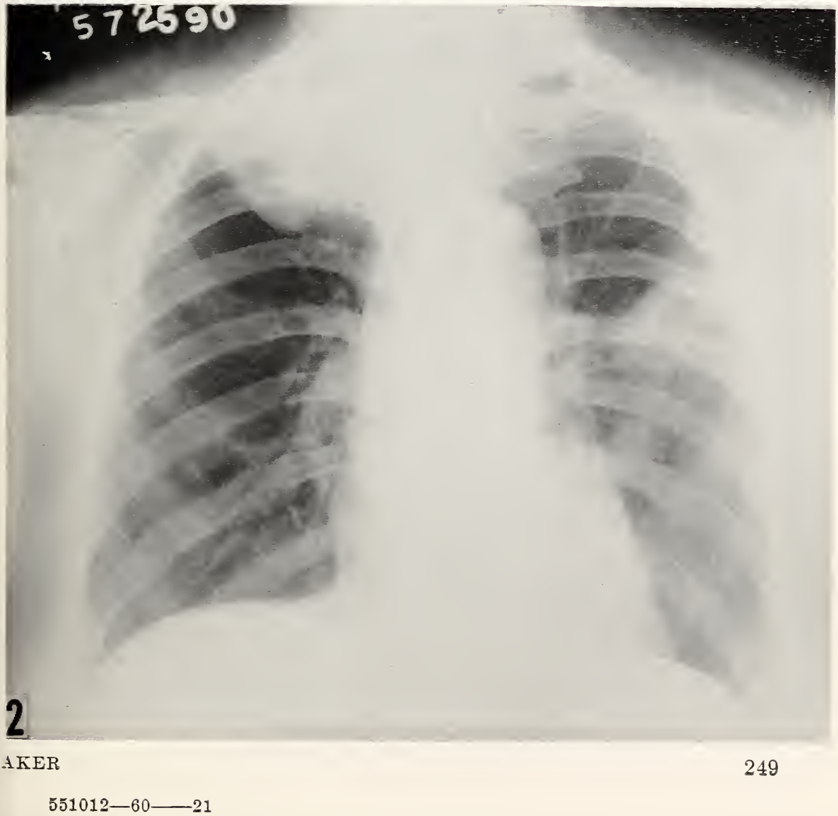

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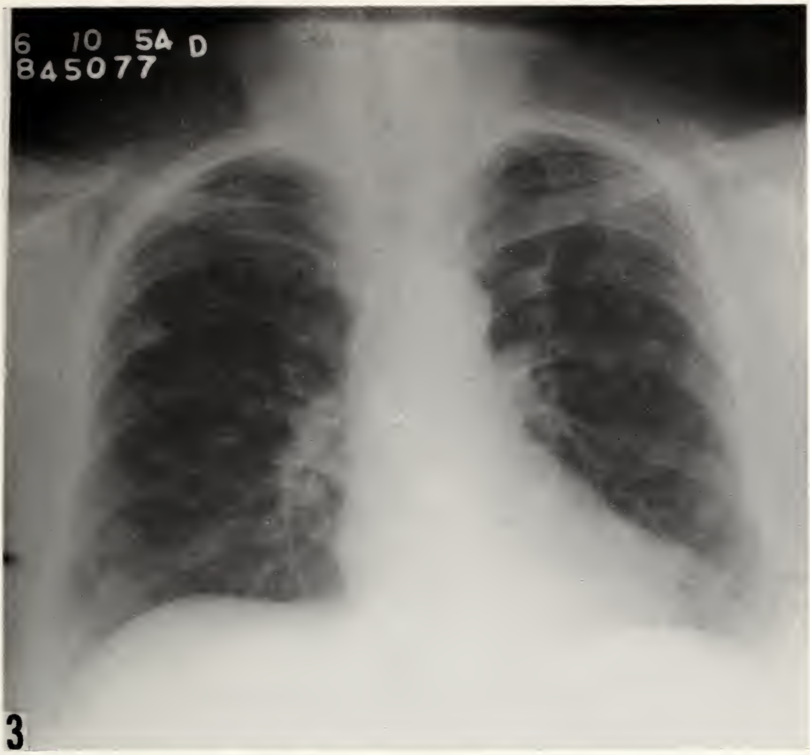
Plate 13

Figure 3.—R.W., MGH 845077, 6/10/54. Multiple nodular densities.

Figure 4.—R.W., MGH 845077, 9/21/54. Slight but definite decrease in most of the metastatic lesions.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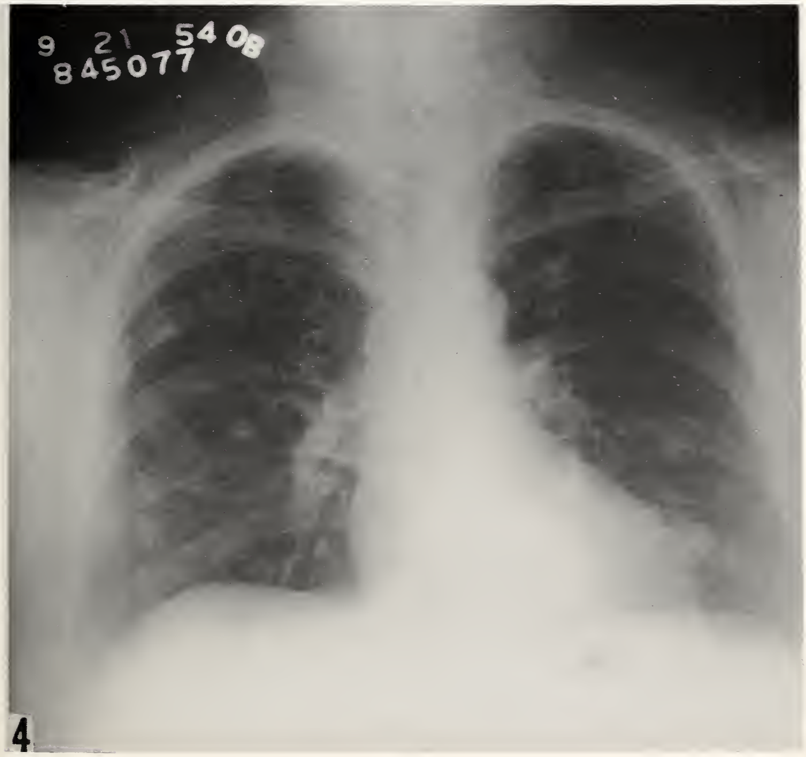
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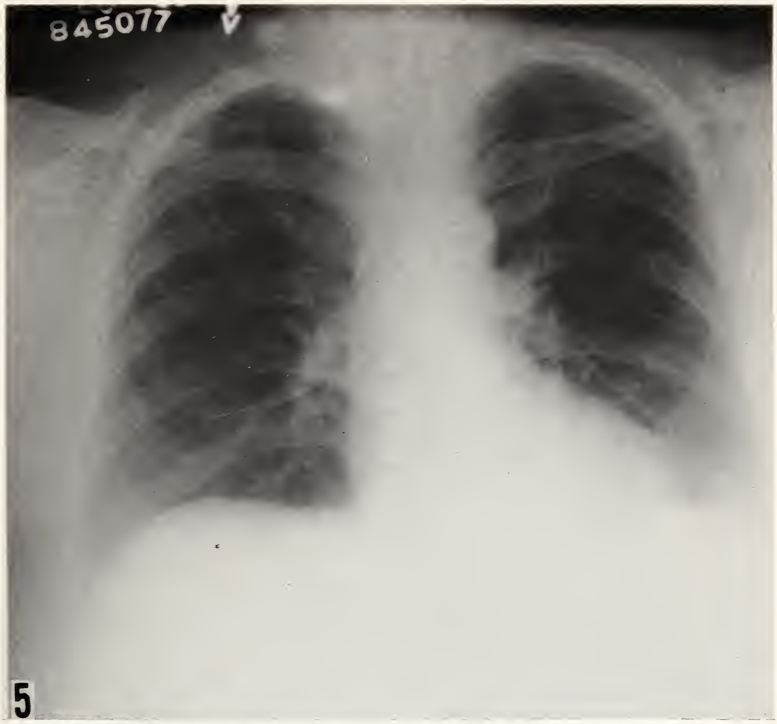
PLATE 14

FIGURE 5.—R.W., MGH 845077, 9/20/55. Regression and disappearance of all nodular densities.

FIGURE 6.—R.W., MGH 845077, 9/24/58. *Note* increased size in hilar area. In *left* lower lung field, adjacent to the cardiac silhouette, *note* reappearance of nodular density.



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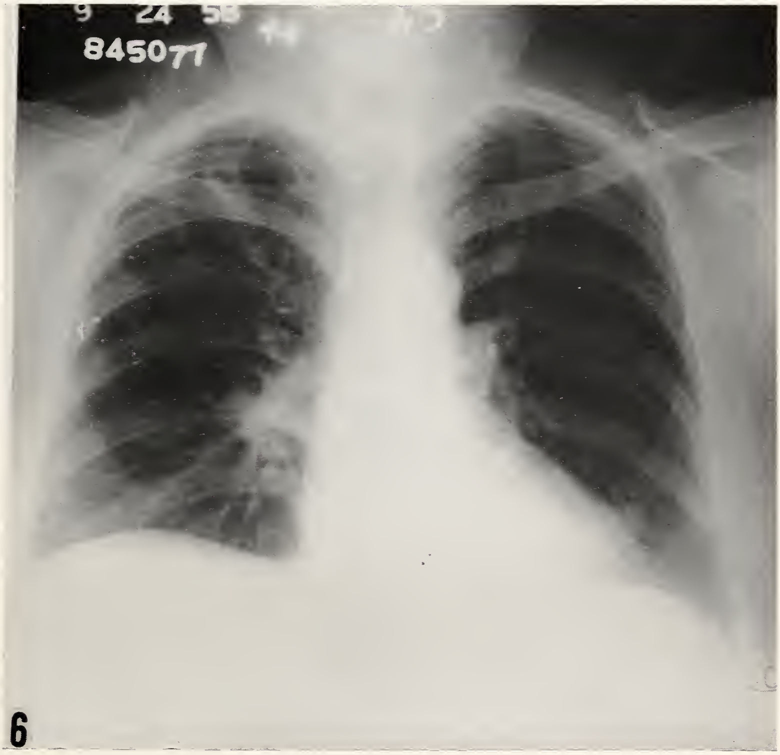
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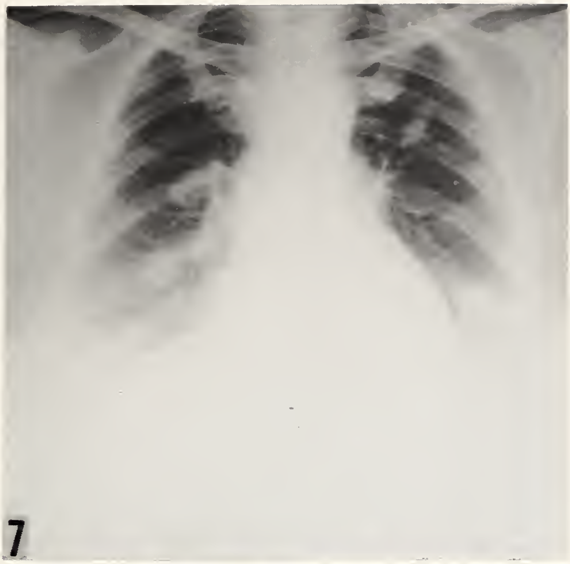
Plate 15

Figure 7.—V.C., PH 39432, November, 1956. Multiple pulmonary metastases in both lung fields.

Figure 8.—V.C., PH 39432, February, 1958. Regression in size of all metastatic pulmonary nodules. Lesions remained the same size up to November, 1958.



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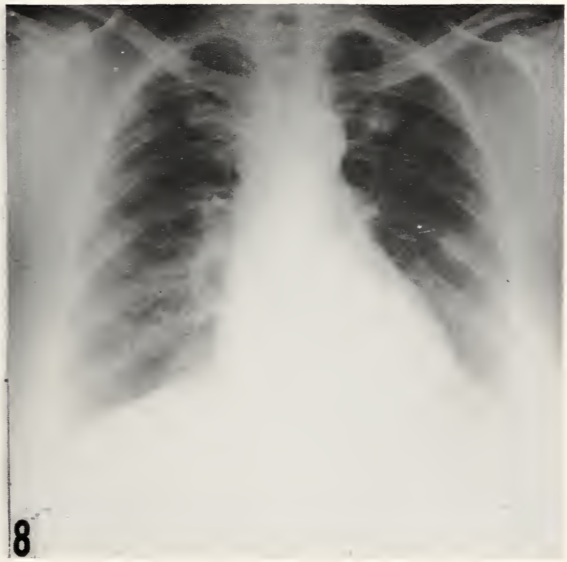

8

Investigations in Breast Carcinoma [1]

Chairman: DR. ALBERT SEGALOFF, *Alton Ochsner Medical Foundation, Tulane University School of Medicine, New Orleans, Louisiana*

Members of Panel:

Dr. Harry F. Bisel, *University of Pittsburgh, Pittsburgh, Pennsylvania*
Dr. Thomas C. Hall, *Lemuel Shattuck Hospital, Boston, Massachusetts*
Dr. George C. Escher, *Sloan-Kettering Institute, New York, New York*
Dr. Rudolf J. Noer, *University of Louisville, Louisville, Kentucky*

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[1] Presented at Conference on Experimental Clinical Cancer Chemotherapy, Washington, D.C., November 11, 1959.

Dr. Segaloff: The largest cooperative study of breast cancer is by the group concerned with hormonal therapy, much of whose completed data I will present.[2]

The protocol which the cooperative group is using was designed primarily to reject compounds significantly less active than testosterone propionate. The use of testosterone propionate as a reference standard has produced a very substantial body of reference data collected in this carefully controlled manner.

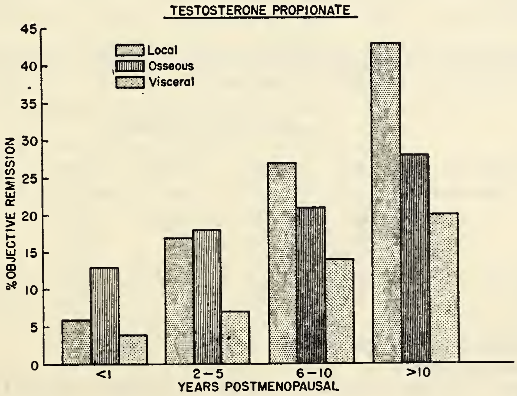
Table 1 shows the distribution of 401 patients, among the dominant lesion and menopausal age groups, treated with testosterone propionate. For each patient we have had at least one follow-up report on the results, but our observations are not necessarily completed on all patients.

TABLE 1.—Distribution of patients treated with testosterone propionate*

	Years postmenopausal				
Dominant lesions	<1	1–5	5–10	>10	Total
Local	18	18	11	53	100
Osseous	30	28	29	65	152
Visceral	26	28	21	74	149
Total	74	74	61	192	401

*Combined data from the cooperative breast cancer group.

Text-figure 1 illustrates that in the different dominant lesion groups and in the different postmenopausal groups there is a distinct pattern, with the response rate getting better with an increase in postmenopausal age and also getting better in the progression from visceral to osseous to local disease.



TEXT-FIGURE 1.—Response rates of patients in dominant lesion and postmenopausal groups treated with testosterone propionate.

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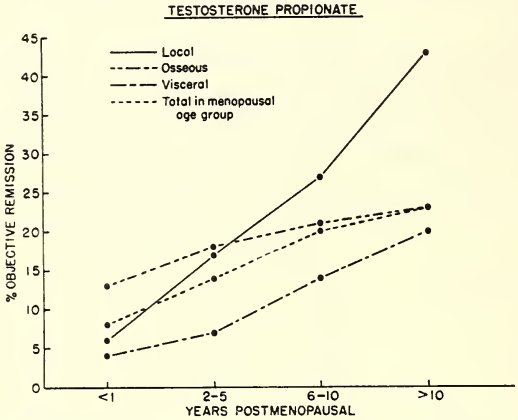
[2] The studies represent the cooperative efforts of members of the cooperative breast cancer group. Statistical consultation was provided by Dr. David Alling, Mrs. Nancy Engel, Mrs. Opal Rasch, and Mr. Marvin Schneiderman. Part of the material was presented at the Conference on the Biological Activities of Steroids in Relation to Cancer, sponsored by the Cancer Chemotherapy National Service Center, Vergennes, Vt., Sept. 27 to Oct. 2, 1959. Academic Press, Inc. In press.

Text-figure 2 shows the data plotted as lines for each dominant disease classification, with the top of the bar graph as the midpoint. Not only is there a significant increase in all these lines with the increase in age, but the lines are not parallel. In other words, the line for the local disease shows a much sharper increase with the increase in postmenopausal age than does the line for patients with dominantly osseous disease or the patients with dominantly visceral disease.



TEXT-FIGURE 2.—Data from text-figure 1, plotted as lines for each dominant disease classification.

As was pointed out from the data and as we had hoped when we set up the study, there is a difference in response rate in these groups. This same pattern tends to hold with the experimental compounds, and we will run through the results with some of the first completed compounds.

Table 2 shows the results of the use of androsterone which produced one remission.

TABLE 2.—Response rates of patients in dominant lesion and postmenopausal groups treated with androsterone*

	Years postmenopausal				Total	
Dominant lesions	<1	1–5	5–10	>10	Number	Percent
Local	0/2	0/2	0/1	0/4	0/9	0
Osseous	0/1	0/1	0/1	0/3	0/6	0
Visceral	0/1	0/1	—	1/5	1/7	14
Total Number	0/4	0/4	0/2	1/12	1/22	—
Total Percent	0	0	0	8		4

*Dose: 100 mg. intramuscularly 3 times a week.
Objective remissions/No. patients.
Albert Segaloff, M.D., and associates, Alton Ochsner Medical Foundation, Tulane University School of Medicine, New Orleans, La.

In table 3, with 5α-androstane-3β,17β-diol diacetate, we see a little better percentage response than with androsterone. However, 2 of these responding patients were in the least favorable groups.

TABLE 3.—Response rates of patients in dominant lesion and postmenopausal groups treated with 5α-androstane-3β,17β-diol diacetate*

Dominant lesions	Years postmenopausal				Total	
	<1	1–5	5–10	>10	Number	Percent
Local	—	0/1	—	1/4	1/5	20
Osseous	0/3	0/2	—	0/3	0/8	0
Visceral	0/1	0/3	1/1	1/3	2/8	25
Total Number	0/4	0/6	1/1	2/10	3/21	—
Total Percent	0	0	100	20		14

*Dose: 100 mg. intramuscularly 3 times a week.
Objective remissions/No. of patients.
Michael Brennan, M.D., and associates, Henry Ford Hospital, Detroit, Mich.

Table 4 shows the 3α stereoisomer of the previous compound, with only a single response in 23 patients.

TABLE 4.—Response rates of patients in dominant lesion and postmenopausal groups treated with 5α androstane-3α,17β-diol dipropionate*

Dominant lesions	Years postmenopausal				Total	
	<1	1–5	5–10	>10	Number	Percent
Local	0/1	—	0/1	0/2	0/4	0
Osseous	0/2	0/2	0/1	0/4	0/9	0
Visceral	0/2	1/2	0/1	0/5	1/10	10
Total Number	0/5	1/4	0/3	0/11	1/23	—
Total Percent	0	25	0	0		4

*Dose: 100 mg. intramuscularly 3 times a week.
Objective remissions/No. patients.
Albert Segaloff, M.D., and associates, Alton Ochsner Medical Foundation, Tulane University School of Medicine, New Orleans, La.

Table 5 shows still another isomer, but here retaining the double bond in the 4-5 position seen in testosterone. This compound is strongly androgenic and has about the same response rate as seen for testosterone. The distribution of the responding patients also does not seem to differ significantly from that for testosterone.

TABLE 5.—Response rates of patients in dominant lesion and postmenopausal groups treated with 4-androstene-3β,17β-diol diacetate*

Dominant lesions	Years postmenopausal				Total	
	<1	1–5	5–10	>10	Number	Percent
Local	—	0/3	0/1	3/4	3/8	38
Osseous	0/3	1/3	0/1	1/4	2/11	18
Visceral	0/1	0/2	0/1	—	0/4	0
Total Number	0/4	1/8	0/3	4/8	5/23	—
Total Percent	0	13	0	50		22

*Dose: 100 mg. intramuscularly 3 times a week.
Objective remissions/No. of patients.
Fred. J. Ansfield, M.D., University of Wisconsin, Madison, Wis., and Kenneth Okon, M.D., Albany Medical College of Union University, Albany, N.Y.

Table 6 shows the response rates with manvine, an estrogen derivative, and here, too, we have somewhat less, but probably not significantly less, response rate than with the reference compound. Interestingly enough, all the patients who responded favorably were in the visceral group more than 10 years postmenopausal.

TABLE 6.—Response rates of patients in dominant lesion and postmenopausal groups treated with manvine*

Dominant lesions	Years postmenopausal				Total	
	<1	1–5	5–10	>10	Number	Percent
Local	0/1	—	0/1	0/4	0/6	0
Osseous	—	—	0/3	0/5	0/8	0
Visceral	0/1	0/1	—	4/9	4/11	36
Total: Number	0/2	0/1	0/4	4/18	4/25	—
Total: Percent	0	0	0	22		16

*Dose: 50 mg. per day orally.
Objective remissions/No. of patients.
Thomas L. Dao, M.D., and associates, Roswell Park Memorial Institute, Buffalo, N.Y.

Table 7 again shows the responses in the greater-than-10-years postmenopausal patients treated with norethynodrel, with about the same response rate as that of testosterone.

TABLE 7.—Response rates of patients in dominant lesion and postmenopausal groups treated with norethynodrel*

Dominant lesions	Years postmenopausal				Total	
	<1	1–5	5–10	>10	Number	Percent
Local	—	0/1	—	2/6	2/7	29
Osseous	0/1	0/2	—	0/3	0/6	0
Visceral	0/1	0/1	0/1	2/4	2/7	29
Total: Number	0/2	0/4	0/1	4/13	4/20	—
Total: Percent	0	0	0	31		20

*Dose: 40 mg. per day orally.
Objective remissions/No. of patients.
Rita Kelley, M.D., Pondville Hospital, Walpole, Mass., and William Baker, M.D., Massachusetts General Hospital, Boston, Mass.

Table 8 shows the response rate with 17α-ethinyl-19-nortestosterone, a substituted testosterone derivative. Again the response rate is about the same as with testosterone. Most of the responses are distributed in the groups as seen with testosterone. This probably does not differ significantly by these criteria from testosterone.

Table 9 shows the response rates with 17α-methyl-19-nortestosterone, which again has a same pattern of response as seen with testosterone. However this compound has a distressingly great propensity for producing jaundice and other untoward symptoms, and was discarded because of its side effects.

TABLE 8.—Response rates of patients in dominant lesion and postmenopausal groups treated with 17α-ethinyl-19-nortestosterone*

	Years postmenopausal				Total	
Dominant lesions	<1	1–5	5–10	>10	Number	Percent
Local	0/2	1/2	—	0/1	1/5	20
Osseous	1/3	—	2/2	2/5	5/10	50
Visceral	—	1/3	0/3	0/5	1/11	9
Total Number	1/5	2/5	2/5	2/11	7/26	—
Percent	20	40	40	18		27

*Dose: 40 mg. per day orally.

Objective remissions/No. of patients.

Isaac Lewin, M.D., and associates, Montefiore Hospital, New York, N.Y.

TABLE 9.—Response rates of patients in dominant lesion and postmenopausal groups treated with 17α-methyl-19-nortestosterone*

	Years postmenopausal				Total	
Dominant lesions	<1	1–5	5–10	>10	Number	Percent
Local	0/1	0/1	1/1	3/7	4/10	40
Osseous	0/1	1/1	0/1	0/3	1/6	17
Visceral	0/1	0/1	0/1	0/2	0/5	0
Total Number	0/3	1/3	1/3	3/12	5/21	—
Percent	0	33	33	25		24

*Dose: 30 mg. per day orally.

Objective remissions/No. of patients.

Anne C. Carter, M.D., and associates, State University of New York, Downstate Medical Center, Brooklyn, N.Y.

Table 10 shows the results with an interesting, steroid-like derivative, Δ^1-testololactone, which has about the same response rate and pattern as testosterone. This compound is being studied further, not because the response rate seems to differ from testosterone, but because it differs from testosterone in having no hormonal activity as far as we can tell. The only apparent biologic activity is the induction of objective remission in breast cancer.

TABLE 10.—Response rates of patients in dominant lesion and postmenopausal groups treated with Δ^1-testololactone*

	Years postmenopausal				Total	
Dominant lesions	<1	1–5	5–10	>10	Number	Percent
Local	—	1/2	—	2/3	3/5	60
Osseous	1/2	—	1/1	1/5	3/8	38
Visceral	0/1	0/2	0/1	1/6	1/10	10
Total Number	1/3	1/4	1/2	4/14	7/23	—
Percent	33	25	50	29		30

*Dose: 100 mg. intramuscularly 3 times a week.

Objective remissions/No. of patients.

Albert Segaloff, M.D., and associates, Alton Ochsner Medical Foundation, Tulane University School of Medicine, New Orleans, La.

Table 11 shows the response rates with 2α-methyl-dihydrotestosterone propionate, which has a different response distribution than testosterone. There are more objective remissions than seen with testosterone and they occur in the osseous dominant rather than in the local dominant group. Because of this, this study is being expanded at the present time.

TABLE 11.—Response rates of patients in dominant lesion and postmenopausal groups treated with 2α-methyl-dihydrotestosterone propionate*

	Years postmenopausal				Total	
Dominant lesions	<1	1–5	5–10	>10	Number	Percent
Local	0/1	0/2	0/2	1/1	1/6	17
Osseous	1/1	3/3	2/2	2/6	8/12	67
Visceral	0/2	1/3	1/2	1/2	3/9	33
Total Number	1/4	4/8	3/6	4/9	12/27	—
Total Percent	25	50	50	44		44

*Dose: 100 mg. intramuscularly 3 times a week.
Objective remissions/No. of patients.
Charles Blackburn, M.D., and associates, The Mayo Clinic, Rochester, Minn.

Dr. Harry Bisel will present some of the data from the group on this type and other types of study with progestational compounds.

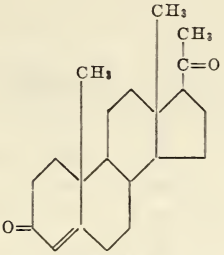
Dr. Bisel: Unfortunately, our results attained with the 2 progestational agents were not comparable to the results Dr. Baker obtained with the 2 progestational agents he used in the treatment of endometrial carcinoma.

The first compound we used was progesterone in oral doses of 2 gm. per day (table 12). The study was conducted at the University of Utah by Dr. Tyler, at the University of Colorado by Dr. Huseby, and at the Yeshiva University by Dr. Volk. The compound was selected in a randomized fashion, compared against testosterone propionate. No objective remissions were obtained with the use of progesterone.

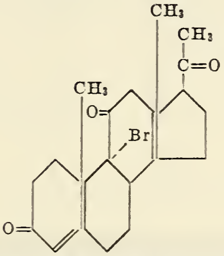
TABLE 12.—Results of study with progesterone, compared with testosterone propionate

	Result		
Treatment	Remission	No remission	Total
Testosterone propionate	6	17	23
Progesterone	0	26	26
Total	6	43	49

The following structures show the structural formula for progesterone and that for 9α-bromo-11-keto progesterone (BOP), which was the other compound used by our group:



Progesterone



4-Pregnene-3,11,20-trione, 9α-bromo (BOP)

Table 13 shows the results of a study with BOP, done in randomized fashion in comparison with testosterone propionate, by Dr. Hays and Dr. Goldenberg at Yale. They obtained 5 objective remissions from 25 patients treated, with a remission rate of 20 percent.

TABLE 13.—Results of study with BOP compared with testosterone propionate

Treatment	Result		
	Remission	No remission	Total
Testosterone propionate	6	18	24
BOP	5	20	25
Total	11	38	49

The Yale group, with 9 other institutions, tried BOP in patients who did not, for one reason or another, meet the protocol criteria. Table 14 shows that, of the 5 remissions observed among the 101 patients, 4 occurred among 21 patients reported by the Yale investigators, whereas only 1 occurred among the remaining 80 patients. The difference between the 2 proportions of remissions, 4 of 21 = 19.0 percent and 1 of 80 = 1.3 percent, is statistically significant at about the 1 percent level. I am indebted to Dr. David W. Alling for this statistical compilation.

Table 14.—Results of BOP study with breast group

Institution	Total	Remission	No remission: Progression	No remission: No progression—treatment stopped
Albany	6	—	6	—
Colorado	4	—	4	—
Einstein	10	—	9	1
Ford	4	—	3	1
Memorial	19	—	18	1
Montefiore	5	—	3	2
Ochsner	10	—	9	1
Pittsburgh	13	1	11	1
State University, New York	9	—	8	1
Yale	21	4	17	—
Total	101	5	88	8

The characteristics of the patients treated at Yale were compared with those of the remaining patients, and these were found to be similar with one exception: 7 of the 21 patients treated at Yale had received hormone therapy other than BOP within 2 weeks of the start of BOP treatment, whereas this proportion was 4 of 80 among the remaining 80 patients. The difference between these 2 proportions, 7 of 21 = 33.3 percent and 4 of 80 = 5 percent, is statistically significant at the 1 percent level.

It is of interest that, of the 4 remissions observed at Yale, 3 occurred among the 7 patients who had received other hormones less than 2 weeks before the start of BOP treatment, and the remaining remission occurred in a patient who had stopped hormone therapy about 3 weeks prior to the institution of BOP.[3]

In conclusion, our results have not compared with the results published in the literature at the dose employed: 100 mg. 3 times a day. The Eastern cancer chemotherapy cooperative study group has also used this compound, and it would be appropriate to ask Dr. Hall to report on their data at this time.

Dr. Hall: The Eastern group has used BOP as part of a series of experiments in breast cancer chemotherapy, one involving the comparative study of nitrogen mustard and thio-TEPA and the other a preliminary study of patients treated with 5-fluorouracil. This was a comparative study of BOP and prednisolone given orally in doses of 300 and 30 mg. per day, respectively. The work was carried out according to a protocol in which the patients' classification by measurement of response was roughly equivalent to that used by the breast groups; therefore the data now to be reported are comparable to those of Dr. Bisel.

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[3] The studies represent the cooperative efforts of members of the cooperative breast cancer group. Statistical consultation was provided by Dr. David Alling, Mrs. Nancy Engel, Mrs. Opal Rasch, and Mr. Marvin Schneiderman.

In this study 31 patients were treated with BOP. Two patients sustained objective responses, a rate of 6 percent. Of the 23 patients treated with prednisolone, 4 percent had objective remissions. Obviously these figures are not significantly different. The patients were, for the most part, more than 1 year postmenopausal. Several showed diminution of the size of the local lesion at the same time that other lesions or ulcerations were progressing; such patients were not defined as responders.

The majority of the prednisolone patients and some BOP patients reported subjective improvement, and the figures are comparable to those reported by Dr. Bisel.

Dr. Segaloff: Dr. Escher will now discuss the role of ablative procedures in advanced breast cancer.

Dr. Escher: A review of the history of the management of advanced mammary carcinoma in women reveals that ablative procedures were first employed more than 50 years ago. In 1886 Schinzinger suggested bilateral oophorectomy as treatment. This was first performed and reported by Beatson in *Lancet* in 1896. Table 15 indicates the results of some of the earlier investigators.

TABLE 15.—Oophorectomy for advanced breast cancer*

Investigator	Date	Number of patients†	Benefited	
Boyd	1900	46	17	
Herman	1901	8	7	
Thompson	1902	72	21	4 "remarkably" 3 "some"
Lett	1905	99	36 31	

*First done by Beatson in 1896, with a complete remission. Data courtesy of Dr. R. J. Noer.

† Seventy-five under 50 years of age.

It is interesting to see the over-all results at the turn of the century. It is, however, more important to analyze more thoroughly Lett's response difference between the 75 under 50 years of age who had roughly a 40 percent improvement as compared with the 24 over 50 years of age who, by the same criteria of evaluation, had half the response rate. Our experience has been even worse, being closer to 10 to 15 percent response rate. This is important because of the recent recommendations for oophorectomy in the older postmenopausal patient. The data presented here do not justify an oophorectomy if the chance of response is poor.

In recent years, the general figures that have been reported vary between 30 and 50 percent objective improvement after bilateral oophorectomy as a therapeutic procedure in advanced mammary carcinoma. This is based, in general, on the criteria used by the cooperative breast group in evaluating objective improvement. Most investigators in this field will accept oophorectomy as the prime method of treatment in the woman less than 1 year postmenopausal who has advanced inoperable mammary carcinoma.

The two areas of dissension that are the subject of considerable discussion are with reference to bilateral adrenalectomy and to hypophysectomy. In a series of 26 patients who were castration failures, none responded to bilateral adrenalectomy. Similarly, in a group of 11 bilateral oophorectomy failures, who were then subsequently treated by hypophysectomy, only 2 showed an extremely transient beneficial effect, neither of which lasted more than 3 weeks. Thus one should think at least twice before considering either of these procedures in the castration failure.

In the patient who is more than 1 year postmenopausal, one cannot derive any information on castration response. The best that I can suggest is that response to androgen therapy may give some hint as to the possible chance of response to subsequent adrenalectomy or hypophysectomy.

Another question that may be raised is: Which is preferable, adrenalectomy or hypophysectomy? In general it is agreed among most endocrinologists in the field that the hypophysectomy patient is easier to manage and that, with some degree of experience on the part of the team handling the hypophysectomy patient, the immediate postoperative as well as the long-term postoperative management is easier.

The reason for making an issue about the use or the rejection of either of these two procedures, in comparison with oophorectomy, is that most of us feel that adrenalectomy and hypophysectomy are much more drastic in the physiologic alteration that they bring about. Therefore one should try to estimate the chances of response much more intensively than when considering the question of oophorectomy.

Dr. Segaloff: The last of our formal presentations, by Dr. Rudolf Noer, will involve probably the most controversial subject in the field of treatment of breast cancer: the question of prophylactic castration.

Dr. Noer: Prophylactic castration might be defined as the elimination of ovarian-functioning tissue by one means or another at the time of primary definitive, operative treatment of carcinoma of the breast. The term prophylactic may not be a good one because theoretically if all the cancer tissue has been removed there is no need for further therapy. People who speak of prophylactic oophorectomy use it in the same sense as those who use the term prophylactic with relation to neck-gland dissection, who do the operation in the hope that some small metastatic nests of cells may be eliminated; with respect to the ovary, that the hormone-dependent cells, which may have been left, may die because of the loss of ovarian hormone.

Prophylactic oophorectomy may be carried out by two means: irradiation or operative removal of the ovaries. Irradiation has been reported to be successful in a considerable number of cases. It has the difficulty of determination of adequate dosage, the need for repeat courses of irradiation to achieve complete castration in many instances, and it also has the difficulty that it is a matter of weeks rather than days before it becomes effective.

In the literature, operative castration has shown a definitely higher incidence of protection against recurrence or metastases. Its effectiveness is immediately apparent and complete as far as elimination of ovarian secretion is concerned.

The interest in prophylactic castration is relatively recent. It is, of course, an outgrowth of the therapeutic castration that has been discussed. There have been some 14 or 15 papers published within the last 10 or 15 years, at least 8 during the last 5, relating directly to the procedure of operative oophorectomy.

Two of these reports are summarized in tables 16 and 17. In the paper by Horsley, analyzing 189 patients with positive nodes, 39 percent of the controls and 63 percent of those who had had oophorectomy were alive at 5 years. Without nodes, the difference was not nearly so great, slightly under 10 percent (table 16).

TABLE 16.—Radical mastectomy, oophorectomy, and hormone therapy*

		Alive after 5 years	
	Number of patients	+ Nodes (*percent*)	0 Nodes (*percent*)
Controls	152	39. 4	63. 2
Oophorectomy	37	63. 2	94. 1

*Horsley: *Virginia Medical Monthly* 78: 226, 1951.

The paper perhaps most frequently referred to as the authority for prophylactic castration is that by Treves which appeared in 1957. In a comparison of controls with castration by irradiation and by oophorectomy he reported these differences: 5-year survivals—39 percent in the controls, 43 percent with irradiation, and 76 percent with oophorectomy. Without positive nodes, the difference was not so great. Ten-year survival again showed no great difference (table 17).

The most recent report published this year by Rosenberg and Uhlman shows a total survival of 59 percent of castrated patients and 36 percent of noncastrated patients.

It is significant that all the evaluation contained in these papers is based upon a comparison of patients who have been castrated by either irradiation or surgery, with control series patients who for one reason

TABLE 17.—Prophylactic castration—mammary cancer*

	Number of patients	Alive after 5 years		Alive after 10 years	
		+ Nodes (*percent*)	0 Nodes (*percent*)	+ Nodes (*percent*)	0 Nodes (*percent*)
Controls	2893	39. 4	77. 4	21. 2	53. 8
Irradiation	84	43. 1	80. 0	23. 3	71. 4
Oophorectomy	68	76. 1	91. 6	40. 0	88. 8

*Treves: *Cancer* 10: 393, 1957.

or another have not received such treatment. As far as I know, there has been no study carried out with a true randomization technique. There has been a difference of opinion among surgeons with respect to the use of castration, as to whether it would perhaps be wise not to castrate at the time of the mastectomy, but rather wait until recurrence appears, if it does, and thus gain for the patient the additional remission which can frequently be obtained by castration at that time.

The disadvantages of prophylactic castration should be mentioned. The first is the operative hazard which is minimal, extremely small in the hands of good surgeons today. The other disadvantage, of course, is the psychologic one. Many women very much dislike the knowledge that their ovaries have been removed. The physical effects of such removal are often not nearly so difficult to control as the psychologic effects.

In conclusion, I would say that a good randomized study of the effects of prophylactic castration is badly needed. I hope that some of the cooperative effort which has developed in the adjuvant chemotherapy program can be utilized to this end.

Dr. Segaloff: I would like to second and third your last plea, Dr. Noer, and since you are chairman of the breast adjuvant group I hope that you will manage to help us along the road in this endeavor. We have several questions to start the discussion. First, Dr. Hall, I understand that possibly you could tell us more about other alkylating agents in breast cancer. We have several questions as to other chemotherapeutic as well as alkylating agents. Could you comment on them?

Dr. Hall: The solid tumor group did undertake, as I mentioned before, a randomized, non-double-blind but independently evaluated study of thio-TEPA and nitrogen mustard in patients with breast cancer. This was at a time when the literature references indicated that there might be a qualitative difference in the action of thio-TEPA with respect to breast carcinoma.

Female patients with measurable disease were treated on a schedule of 4 daily injections of the agent followed by a 2-week rest period with subsequent weekly doses for a total treatment period of 90 days. The single dose levels were 0.1 mg. per kg. for nitrogen mustard and 0.2 mg. per kg. for thio-TEPA. In the 37 patients treated with nitrogen mustard 7 objective remissions were noted. These involved soft tissue and bone. There was an average decrease in tumor size of 44 percent and the average duration of response was 36 days, though treatment on the average was continued for 74 days.

In the 39 patients treated with thio-TEPA, the objective response rate was 8 percent, that is, 3 patients. The average decrease of the tumor size was 30 percent; the average duration of response was 45 days, though treatment was continued for approximately 82 days. The toxicity of the 2 compounds was essentially the same. The patients were identical in respect to previous therapy and extent of disease, and the results did not support the hypothesis that thio-TEPA was an unusually effective or qualitatively different alkylating agent in breast carcinoma.

Since you have referred to an interest in other types of chemotherapy, may I mention our initial experience with 5-fluorouracil. This compound was used at first in a small series of 9 cases of breast cancer, all of whom received a dose equivalent to that suggested by Curreri *et al.*, or sufficient to produce marked bone marrow responses. Only 1 patient responded with changes in soft-tissue disease. Subsequently 17 patients have been treated at the Lemuel Shattuck Hospital; 3 of these had objective responses persisting for an average of 3.5 weeks.

Dr. Segaloff: We have several questions from the floor that I would like to pose: In view of the data that look so nice for prophylactic castration, would we do it? Suppose we poll the panel. Dr. Hall.

Dr. Hall: There is considerable interest in doing this in our geographic area and we have enlisted the services of Dr. Bryan McMann, Professor of Epidemiology at the Harvard School of Public Health, to help us design the protocol to study this in the Boston area over a 5-year period. I think the answer is yes.

Dr. Segaloff: Dr. Bisel.

Dr. Bisel: I am pretty hard to sell on this point but still I am very much in favor of seeing an informative study conducted and I hope that with Dr. Noer's support perhaps the breast adjuvant study group will get the ball rolling.

Dr. Segaloff: Dr. Noer.

Dr. Noer: We are not doing it at the present time because we are participating in a common protocol aimed at arriving at results on adjuvant chemotherapy so we shall stick with the protocol until the uptake of 600 cases is finished, we hope by about July 1960. And I hope at that time we can participate in a group effort using the randomization technique to study the value of prophylactic castration.

Dr. Segaloff: I suppose I can vote next. I do not believe in carrying out prophylactic oophorectomy at the present time but would like to both see and participate in a study as mentioned by Dr. Noer. Dr. Escher.

Dr. Escher: I have been saying yes since 1951 and because I have been trying to get a randomized study started since then, I vote yes.

Dr. Segaloff: The next series of questions tie in with this: Dr. Escher, how would you decide whether or not to do a hypophysectomy in terms of response if the patient had had a prophylactic castration and then had recurrence?

Dr. Escher: Would adrenalectomy or hypophysectomy be indicated for recurrent disease in a woman who had prophylactic oophorectomy? I would prefer to recommend first the use of androgens. It is much simpler to give and stop androgens than to do a hypophysectomy. Next, would you recommend prophylactic oophorectomy in a premenopausal woman with operable cancer of the breast? The answer is yes.

How about hypophysectomy in patients who have had remission after castration and then relapsed? Here, too, the incidence of response to administered androgens is higher if the patient has responded to castration, and we would like to recommend that this be the next course of therapy and that the hypophysectomy be reserved as a subsequent procedure with the odds of response to the hypophysectomy somewhere in the vicinity of 30 to 40 percent.

Dr. Segaloff: Dr. Noer, would you recommend a hysterectomy at the time of oophorectomy?

Dr. Noer: No, I would not. I am afraid I may not get what is behind the question because it does not occur to me that this is a matter for decision.

Dr. Escher: May I interject a comment? One of the things behind that question is the issue as to whether the uterus, the endometrium, or some other portion of the uterus is the source of a hormone. This issue has been raised but no one has resolved it.

Dr. Noer: Do you want to comment on that, Dr. Segaloff?

Dr. Segaloff: The rabbit endometrium puts out something, but we do not know anything about the human female. Is there any other comment?

Dr. Hall: There is an unfinished study from Kings County which relates the beneficial effects, *i.e.*, duration of life and interval between operation and appearance of metastases to the time of menopause, not to the time of cessation of ovarian function as measured by other maneuvers. This just shows a high degree of positive correlation and there certainly is room for more data.

Dr. Segaloff: We have several other types of questions that are addressed to the panel in general, some of which I would like to comment on unless the panel would like to disagree. One of them is: Discuss the use of ablative procedures other than oophorectomy in the patient with metastases at the time of mastectomy. I would like to pass this one to Dr. Noer because I disapprove of doing other ablative procedures and surgery at the same time.

Dr. Noer: I would not do both at the same time. This is a patient with metastases, by the wording of this question. I would not do any ablative procedures at the time of the operation, preferring to gain the succession of remission, referred to by Dr. Escher, by subsequent ablative procedures.

Dr. Escher: If the patient has metastases, why do the mastectomy?

Dr. Noer: This is a good question. One might say that there would perhaps be one indication for doing the mastectomy in a patient with a large lesion threatened with ulceration and a stinking mess to live with the rest of her life. I believe this would be justifiable.

Dr. Segaloff: Dr. Noer, as the surgical member of this panel, and along the same line, what is the recommended therapy for a pregnant woman found to have breast cancer? Is abortion indicated in the first trimester? What is to be done for a woman 3 to 4 years after mastectomy who becomes pregnant? Is abortion indicated?

Dr. Noer: This is a difficult question because we all know that many of these carcinomas tend to accelerate their growth tremendously in association with the hormonal influences associated with pregnancy. A great deal depends upon the individual patient. There are many patients who would not allow an abortion. I think one might seriously consider whether this is desirable.

Pregnancy 3 or 4 years after mastectomy is a matter which has been discussed in a number of interesting papers in recent years. Most people feel that a woman who has had carcinoma of the breast should not be allowed to become pregnant, again because of the hormonal influence which might be brought to bear. There are, however, a few competent individuals in the profession who have said that they believe it is justifiable for a woman who has gone 3 or 4 years without recurrence to have children and take the chance on a possible activation of a few cells which might be present somewhere. Perhaps some of our endocrinologists like Dr. Segaloff might have some additional comments.

Dr. Segaloff: I would rather not add anything. I agree thoroughly. The next question is addressed to Dr. Bisel: Is there any place for the use of isotopes such as P^{32} in the treatment of advanced breast cancer?

Dr. Bisel: I am sure there is a place for isotopes such as P^{32}, chromic phosphate, or colloidal gold in the treatment of the serous effusions resulting from advanced breast cancer. Beyond that, I am not prepared to say that there is a place. The person who asks this question may have in mind the work of Maxfield, the late Dr. Low-Beer, Dr. Bell, and others, who have written about the use of P^{32} as an adjuvant to surgery and its use systemically. Certain advanced breast carcinomas selectively pick up radioactive phosphorus in greater concentration than normal tissue, and in certain people's hands, with and without the additional use of an androgen, some results may be obtained in skin metastases and in bone metastases. I do not think any claims have been made for its use in visceral metastases. P^{32} has been used, to some extent, at our own institution. It is a procedure that should be thought of after hormonal and other systemic treatment has been tried and failed, because by and large the results obtained have not been as good as with other modalities at our disposal.

Dr. Escher: Most of the studies to date report increased uptake of P^{32} after pretreatment with testosterone propionate, but no one reports any comparison with pretreatment uptake.

In an attempt to answer the question more accurately, a woman with recurrent mammary carcinoma with an osteolytic lesion in one femur and with no obvious lesion in the opposite femur, who was postmenopausal and had received no previous hormone therapy, was given a tracer dose of Sr^{85}. Uptake was measured over the lytic lesion in the one femur and over the same area in the other uninvolved femur. The patient was given testosterone propionate, and we waited until there was a distinct flare of alkaline phosphatase, which is supposed to indicate enhanced uptake of whatever one would expect would repair bone. Then another tracer dose of strontium was given. There was no evidence at all of any enhancement of uptake of strontium. For this reason, and after conversations with Dr. H. Friedell who first did this study and who dropped it, we feel that this technique is at best of limited value.

Dr. Segaloff: One of the questions directed at me is: Is it true that there are now more recent data, accumulated on Δ^1-testololactone and 2-methyldihydrotestosterone, which have not entirely substantiated the initial results? If it is true, I am not aware of it. The data that were presented at the cooperative breast group meeting are on patients who have previously had other hormonal therapy and were not suitable for treatment under the protocol and therefore they are not at all comparable to the data which were presented just now.

I have been asked to comment on the report of Dr. Ian Macdonald concerning the American Medical Association breast study group findings which indicated that, in the 3-year-plus postmenopausal group, estrogen was twice as effective as androgen in all sites and in each age group above 3 plus menopausal. Since I participated in the study, I have at least the license to say that the studies here presented for the cooperative breast group with testosterone are the first adequate studies undertaken with adequate randomization, adequate protocol for the entry of the patients, and adequate follow up. That is all I care to comment about the AMA studies, particularly since I do not know the results to be reported.

The next question is also addressed to me: What is your estimate of the correctness of the conclusions reached in a recent large series reported with the use of irradiation castration? I presume that this is the series reported from Manchester which showed that with prophylactic irradiation castration there did seem to be a short delay in the onset of first metastases, and an increase in the patients alive at 5 years. This is an important finding and I am in favor of doing more and better studies with prophylactic castration. However, I would rather use as controls a group of patients castrated at the onset of metastases than a group that had not received such therapy. Dr. Escher has mentioned that 40 to 50 percent of patients undergoing therapeutic castration get substantial objective remission. Such a group should be paired with another group undergoing prophylactic castration. This study would yield interesting answers to the question of whether the benefits stemming from prophylactic castration exceed the benefits demonstrated as a result of therapeutic castration.

There are two other questions that are addressed more generally and we will ask if the panel can answer either of them. Does anyone have any experience with the successful treatment of advanced cystosarcomaphyllodes with a chemotherapeutic agent? I do not. The answer, then, is no. Is there any proof of enhancement of radiation sensitivity of breast cancer by any chemotherapeutic agent? If so, what drugs, dose, and in which patients? Most of us working in breast cancer and particularly in hormonal areas of breast cancer feel greatly indebted to the late Dr. Nathanson who originally tried to show that estrogen produced an increased radiation sensitivity, but he was unsuccessful in doing this. I am not aware of any other agents showing that you could increase radiation sensitivity. Maybe someone in the panel has some information.

Dr. Escher: I wonder if this refers to some of the reports on the increased radiation sensitivity following actinomycin D, based on the fact that you get an erythema earlier when you have given the actinomycin. I wonder whether it is not more the fact that these tissues are being injured by two agents and the erythema is just evidence of further damage rather than enhanced sensitivity to radiation therapy.

Dr. Hall: There was a small series done at Pondville Hospital in which inflammatory carcinoma of the breast, treated with estrogen followed by X irradiation, seemed to have a better, probably just barely statistically significant (not double-blind, not controlled), long-range outcome than non-estrogen-treated irradiated cases and nonirradiated estrogen-treated cases. This was a retrospective study in a small number of cases with one subspecies of breast carcinoma.

Dr. Segaloff: Dr. Noer, when are we going to randomize simple versus radical mastectomy?

Dr. Noer: The real difference between the simple and radical mastectomy is that the simple mastectomy removes the breast tissue alone, and the radical mastectomy removes the first line of tributary lymph glands.

The studies of McWhirter and others have cast some doubt upon whether a radical mastectomy is greatly superior to simple mastectomy. However, all surgeons today feel that the former is definitely superior, and I believe that they have some basis for this belief in the finding of positive nodes in a patient's axilla, after the dissection of the axilla as part of a radical mastectomy, and in a considerable percentage survival of patients, despite the positive nodes which have been removed from the axilla. Most of us doubt the efficacy of irradiation to sterilize those nodes in the axilla. I think there is perhaps an even greater need for a controlled study of postoperative irradiation than for a controlled study of prophylactic castration. I am told by the statisticians that these two could be combined in one study. That is the study which I hope will be the next one and which, if I have anything to say about it, will probably precede simple mastectomy, for I think we will have even more trouble getting surgeons to refrain from doing radical mastectomy in the control series than we have had in finding institutions which would omit postoperative irradiation so that for our chemotherapy project we might have controls in patients with axillary metastases.

Dr. Segaloff: Dr. Escher, do you know of any evidence that hypophysectomy can and will give better objective or subjective remissions than treatment with available hormones or other drugs?

Dr. Escher: I will say no and then I will try to explain. If one takes the figure of 50 percent response at best to castration, and then takes that 50 percent group of patients and treats them with hypophysectomy, the best objective response that one can expect is somewhere in the vicinity of about half those patients. That gives a figure somewhere in the vicinity of 25 percent in the over-all group. We have told you that in a nonselected group of patients ranging from less than 1 year postcastration through more than 10 years postmenopausal, the over-all response rate is 20 percent. I therefore think we are fair in saying that hypophysectomy is not significantly better than any additive hormone.

Dr. Segaloff: The last question is: Approximately what are the chances of response to androgen therapy when metastatic disease has progressed after oophorectomy in a previously menstruating woman? The answer to this is the data that we have cited on the testosterone responses of patients. There is no categorical answer to this question. As far as we can tell from the data that we have presented, the chances of response depend on the site of major involvement with lesions and on how long after oophorectomy the patient goes before she has advancing disease which requires further treatment. If she goes a long time, she has a better chance of responding. If she has a local recurrence instead of a recurrence in her lung or liver, she has a better chance of responding. If she has osseous recurrences, she has an intermediate chance. The chances of response, at least to testosterone and to many other hormonal agents, depend entirely upon what area is involved with the metastases and how far postmenopausal the patient is.

Newer Techniques and Some Problems in Cooperative Group Studies[1]

Chairman: DR. C. GORDON ZUBROD, *National Cancer Institute, Bethesda, Maryland*

Members of Panel:

Dr. Johannes Ipsen, *Massachusetts Institute of Laboratories, Boston, Massachusetts*
Dr. Emil Frei, *National Cancer Institute, Bethesda, Maryland*
Dr. Louis C. Lasagna, *Johns Hopkins Hospital, Baltimore, Maryland*
Dr. Mortimer B. Lipsett, *National Cancer Institute, Bethesda, Maryland*
Dr. Edmund Gehan, *Cancer Chemotherapy National Service Center, Silver Spring, Maryland*
Dr. George C. Escher, *Sloan-Kettering Institute, New York, New York*

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[1] Presented at Conference on Experimental Clinical Cancer Chemotherapy, Washington, D.C., November 11, 1959.

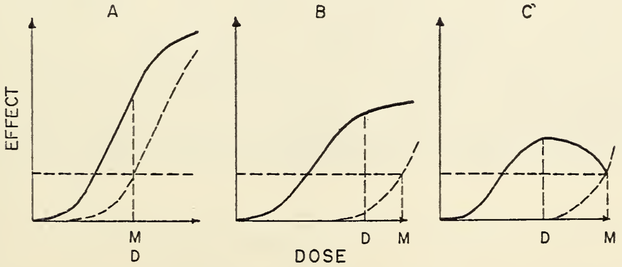
Dr. Zubrod: I have invited most of the members of the Experimental Design Committee to sit on this panel and, in addition, have asked Dr. Frei and Dr. Lipsett to cover some special problems. The members of the panel have met several times to discuss what might be covered in the dying hours of this meeting. We decided to select some of the controversial points which have been mentioned repeatedly during the conference and to make some comment about them.

The first difficulty has to do with the concept of the maximum tolerated dose. Many of the physicians dealing with cancer feel that to give a drug a fair trial the maximum tolerated dose for the patient should be used. Dr. Lasagna and others have raised some questions as to whether the maximum tolerated dose was always the optimal way of giving a drug.

Dr. Johannes Ipsen will speak on this point.

Dr. Ipsen: I am speaking as a member of the Committee on Experimental Design, and I am not actually engaged in clinical work with cancer. Last summer our chairman asked us whether there was any way to utilize better the information that comes out of phase-2 studies when a new drug with unknown efficiency and unknown side effects is applied to patients for the first time. The object of phase 2 is to determine the human effective dose and/or the maximum tolerated dose.

According to the "doctrine of the maximum tolerated dose," the only ethical procedure is to use the maximum tolerated dose as the therapeutical dose because it is believed that, with increasing dose, the side effects and the beneficial effects will rise to the same extent. Text-figure 1 is a diagram that represents 3 situations with beneficial effects and side effects related to increasing dose. Diagram A illustrates that the side effects and the beneficial effects are parallel. The dose of choice in this situation is the maximum tolerated dose, since less effect would appear if a lower dose were given.



Text-figure 1.—Diagram (A, B, and C) representing beneficial effects (*full line*) and side effects (*broken line*) related to increasing dose. The *horizontal broken* line represents a maximum tolerated level of side effects. M is the maximum tolerated dose; D the therapeutic dose of choice.

However, there can very well be the system, as shown in Diagram B, in which the two curves are not parallel. The therapeutic effect is a leveling off over a dose range where the side effect is rising sharply. Here the dose of choice, or the ethical choice, is not the maximum tolerated dose but rather the dose at a point of minimum return of therapy.

Finally, in Diagram C we have a situation mentioned as a prozone effect, which was postulated not to exist in certain types of drug-cancer systems, but of which there are many examples in other drug-disease systems. The curve of effectiveness is approaching an optimum rather than an asymptote, and this optimum is placed at a much lower dose level than that causing side effects. If the optimum can be established, it would be highly unethical to give the maximum tolerated dose, since less effect is attained by this dose than a much smaller one.

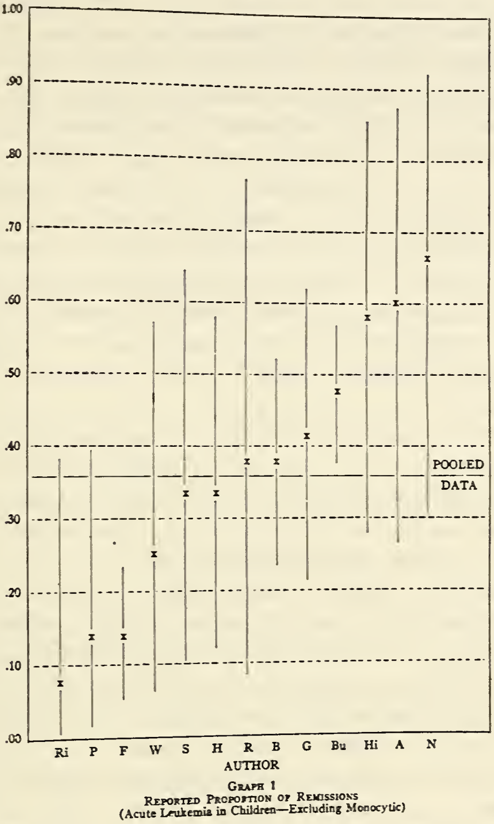
The point for discussion is, then, the following: In phase 1 can the experiment be designed so that the two regressions on dose are displayed? One would hope to attain this by designing phase-1 studies on multiple-dose levels. How this could be attained in practice I will not go into because it would depend on the disease and the drug in question. The essential part is, however, that a certain dose level is maintained in each patient rather than the "sliding dosage" which is often practiced in phase-1 studies. The maximum tolerated dose may not be the dose of choice.

There are 2 other benefits that may come out of the multiple-dose design. (1) A regression of effectiveness on dose in itself is an internal control of effectiveness. Phase-1 designs typically do not include "untreated" control groups. (2) There could be a weighing of the relative importance of the many clinical signs, symptoms, and measurements that are used to evaluate outcome and prognosis. Many of these observations are currently given predetermined scores that are added to give a measurement of either prognosis or outcome. There are statistical methods available that permit a more objective scoring by relating dose levels and frequency of symptoms. This can be done even on a small group of patients so that a multiple-dose phase-1 study would prepare the design of the final phase-3 study, not only in respect to choice of dose but also in indicating which observations are the most informative.

Dr. Zubrod: Another problem which has been referred to repeatedly has been that of criteria: criteria for diagnosis, as shown by Dr. Robert Lukes, criteria for remission, criteria on every score. I think it is clear that unless we are all talking the same language it is difficult to make any kind of comparison of results. Because of some of the difficulties which Dr. Freireich mentioned on the leukemia panel, I have asked for further discussion of the problem of the criteria in leukemia by Dr. Emil Frei.

Dr. Frei: Text-figure 2 presents the remission rate in 13 chemotherapy studies, which were conducted by various investigators, in children with acute leukemia. The remission rate ranged from less than 10 to almost

70 percent. In those studies involving fairly large numbers of patients, the confidence limits did not overlap, and the difference between the results in terms of remission rate was highly significant.



Text-figure 2.—Remission rates in 13 chemotherapy studies. The "*X*" marks the remission rate achieved, and the *solid lines* the 95 percent confidence limits of these remission rates. (Reprinted from *Ann. New York Acad. Sc.*, Dec., 1954.)

Remission rates in the area of 10 percent for children with acute leukemia probably approached those which would occur spontaneously. Thus, investigators reporting a 10 percent remission rate may have concluded that the drug (6-mercaptopurine) was inactive, whereas those reporting a remission rate in excess of 50 percent were obviously dealing with a highly active compound. These studies were compiled by Bross from the meeting on 6-mercaptopurine held by the New York Academy of Sciences in December, 1954.

Why were the results of these studies so different? If one analyzes the method of use of 6-mercaptopurine, it is apparent that the methods of administration of the drug to the patients were essentially the same. The investigators involved were all highly competent and familiar with chemotherapy, particularly with the chemotherapy of acute leukemia. There are a number of factors which determine these differences in response in terms of remission rate. But in analysis, overwhelmingly, the most important factor was the criteria for remission used by the different investigators.

I have presented this study to make what I believe to be one of the most important points concerning criteria for response to therapy: The application of widely differing criteria can result in very different interpretation of experiments that are essentially similar. One might answer that response should never be evaluated in the absence of detailed analysis of the author's criteria. For many reasons this is often not done.

In scientific endeavor it is important to fan out, to define new areas, new ideas, and to explore these. But as this is done, in a given area, it is also important that our means of communication come closer together. There is ample scientific as well as political precedent that progress in part depends on sharpening up our method of communication. This is what criteria for response to therapy really are, not only from one to another in terms of remission rate but also within one's own studies over a period of time.

Considerable effort concerning criteria for response to therapy in acute leukemia has been made over the past 4 years, and it is probable that the highly varying remission rate for a given drug in 1954 would not occur today. In other words, our means of communication are somewhat better. However, several problems still exist.

I have emphasized the need for uniformity of criteria. It is, of course, of primary importance that they be correct in terms of applicability, etc. While it is necessary to be arbitrary in the relation of many values for criteria, there are certain principles on which these can be based. There is general agreement that complete remission values ought to be defined in terms of the normal. There is also agreement that the criteria should be sensitive enough so that minor but significant degrees of response will be recorded. Between these extremes the points of separation of degrees of response must, in large measure, be selected arbitrarily. I think it might be appropriate at this point to invoke Parkinson's Law as Dr. Rundles did: "A judicious and arbitrary decision is better than endless discussion and argument."

These factors apply to other forms of cancer as well as to acute leukemia. The application of uniform and effective criteria for response would in no way impede research and would, on the contrary, facilitate communication and interpretation of data and thus save time for the more stimulating and important areas of therapeutic research.

Dr. Zubrod: A third problem which has arisen in almost every panel has been: What are doctors going to use to prove that a good therapeutic response has been achieved? Often with a good deal of emotion, doctors feel that their total experience tells them that one parameter is the important thing in describing the improvement which occurs in using drugs in patients with cancer. The two that have had the most attention, of course, in the last 2 days have been survival of the patient and size of the tumor. Dr. Lasagna will make some comments about this general problem.

Dr. Lasagna: First some comments about the objective and subjective classification of criteria. In many instances this is erroneous, because wherever there are humans involved in making measurements, there is a good deal of subjectivity involved. One common implication is that double-blind techniques are not needed for so-called "objective" measurements, whereas they are for "subjective." Dr. Chalmers touched on this point but I will remind you that, wherever studies have been made of the reading of X rays "blind" by radiologists, it has been shown that there is considerable error and bias in repeated measurements over time (even by the same individual of the same X ray).

I have been a bit distressed by the facetious references to the double-blind techniques, not because I think the people who made them really had doubts about the technique but because they do lend fuel to the fires of those poor, benighted members of the medical "booboisie" who have not realized the advantages to be gained from the double-blind technique in eliminating bias and prejudice and controlling for spontaneous change. Without the presence of a placebo, for example, in the cooperative Veterans Administration lung hormone study, the results would have been almost uninterpretable.

Next, some comments about the concept of quantal or global criteria as opposed to continuous or quantitative measurements. It is all right for investigators to decide on criteria that take into account the totality of response (*e.g.*, both the beneficial and the toxic reactions seen) in determining that a drug produces 50 or 20 percent "satisfactory" responses. However, in addition to this type of information in reports, I would plead for a detailed breakdown of the individual parameters that have been studied, because otherwise a great deal of information is lost. Only a detailed breakdown of what has been satisfactorily achieved and what has not will enable people to get the most information from these studies.

I am heartily in favor of the use of different parameters. Reference has been made to the use of tumor size and survival time. In regard to survival time, I wish that more studies would employ the life-table approach, which has not always been utilized in the studies reported at this meeting. The use of tumor size in this area is like the use of blood pressure in the study of hypertensive cardiovascular disease. Everyone will admit that one can lower the blood pressure with drugs and yet either not benefit the patient with hypertension, or perhaps even cut short his lifespan. The same certainly may be true about causing decreases in tumor size in the patient with cancer. Yet it is hard to envision that an agent that is really successful in treating hypertensive cardiovascular disease will not lower the blood pressure, or that an agent that is really effective in prolonging the lifespan of people with cancer will do this without affecting tumor size.

Tumor size is seized upon not only for this reason but also because the study of survival time is quite complicated and becomes more complicated when there is a long interval elapsing between the administration of the treatment under study, and death. The more time that elapses the more chance there is for other treatments to "get into the act" and the easier it is to confound matters and to be confused as to just how much the original drug has contributed.

I am in favor of using gonadotropin titers, measurements of Bence Jones protein in the urine, acid phosphatase in the serum, or any other such parameters as seem relevant to the studies in question. I am also in favor of studying subjective phenomena, such as pain and well-being. We pay all too little attention to these points that are often more important to the cancer patient than what is happening to a nodule in one lung field.

The point is perhaps worth making that the clinical studies that are being conducted are for the most part difficult, take a long time to execute, and are not terribly precise. Even if they were, however, it would be unrealistic to expect a clinical study to pick up very slight differences in activity between drugs. One can easily calculate, for any given percentage difference between treatments—depending on what the standard treatment does and what is expected in the way of betterment of this standard performance—how many patients are required for a given study. If one does this, it is often appalling to note the large numbers of patients that are needed to show up small differences between drugs. Thus if the chemists are looking for this type of information, they are probably not going to get it.

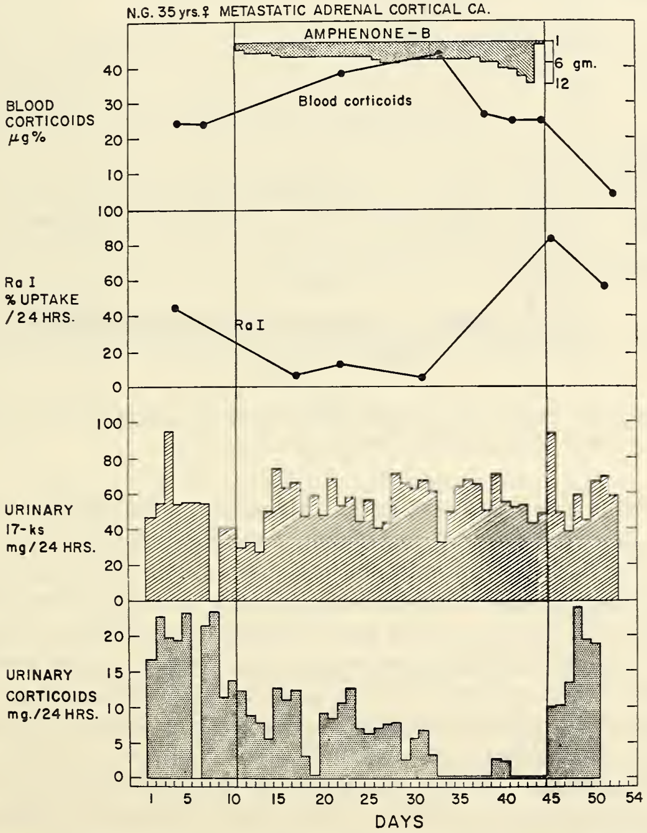
A corollary of this is that, in choosing drugs for study, one would want to select new agents that appear more promising in a variety of screens than the standard drugs. I say this (1) because we might then have a better chance of picking up compounds that are significantly different from standard drugs, and (2) if such a promising compound turns out to be ineffective, it has a great deal more meaning for our interpretation of the screens than if we study a compound that is only slightly better and perhaps only better in one particular tumor screen.

Dr. Zubrod: It is quite apparent that we do not have a satisfactory measure of the behavior of the total tumor mass in patients. We, at best, observe a small fraction of the tumor and, at best, by indirect means can guess what the behavior and growth or regression of this tumor might be. One type of measurement that would help considerably would be a characteristic chemical product of a tumor, easily measurable and excreted in proportion to the size of the tumor. Dr. Mortimer Lipsett will discuss this problem.

Dr. Lipsett: The discussions of the previous panel, and of many other panels, are a tribute to the difficulties of assessing the effectiveness of any chemotherapeutic drug. As Dr. Lasagna pointed out, the number of patients that is needed to distinguish small differences in activity is rather large, yet these small differences can be quite important in providing a clue for research in a particular direction. To get this clue without using a large human screening program, it is advantageous to work with tumors that produce a measurable end product or with tumors that cause some alteration of metabolic activity of the host.

Thus, if there is a measurable parameter of tumor activity, it is possible, very quickly, to get an indication of a chemotherapeutic effect. Such indications do not mean, however, that the chemotherapeutic agent is necessarily affecting the growth of the tumor. For example, one can certainly disassociate the production of hormones by an adrenal cancer from inhibition of growth or from the death of the tumor cell.

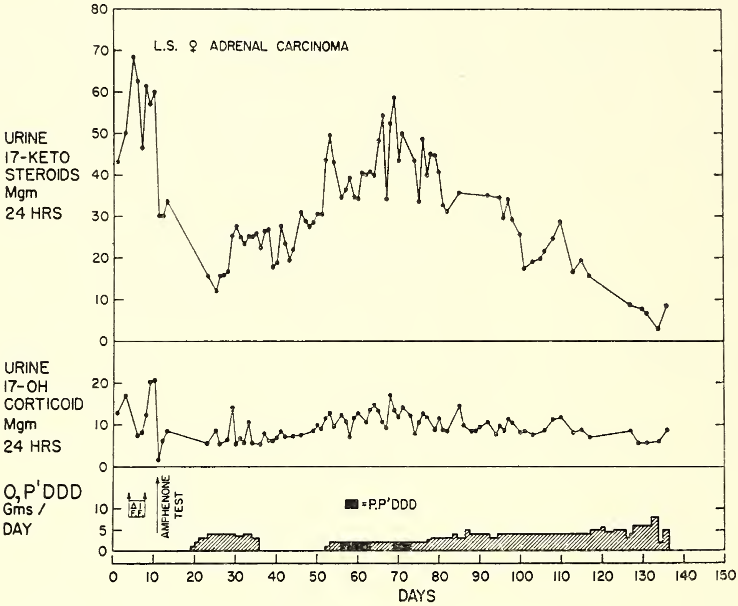
Text-figure 3 represents a study with amphenone, which produces a sharp drop in the excretion of hydroxycorticoids, ketosteroids, and other measurable steroids. It does this in the patient with normal adrenal glands and in the patient with adrenocortical carcinoma. It was initially hoped that the drop in secretions by the adrenal cancer cell would reflect a slowing of the rate of growth of the cancer, but this did not prove to be true. This, then, is an example of an instance in which reliance on changes in a secretory product of the tumor cell did not reflect correspond-



Text-figure 3.—Study of the use of amphenone in a patient with metastatic adrenocortical carcinoma.

ing changes in the growth of the tumor cell. However, the patient with adrenocortical carcinoma does have a convenient "handle" for assessing a number of antitumor agents.

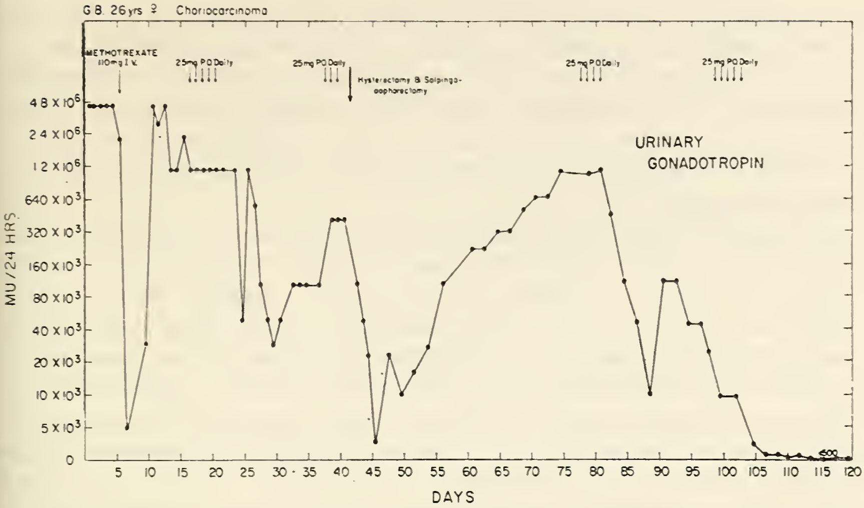
Text-figure 4 is a study of the first patient that was treated with ortho, para prime isomer of diphenyldichloroethane (*o,p′*DDD). When this study was started we had only small amounts of material available. Initially there was a drop in urinary ketosteroids, even with this amount of DDD. When the drug was stopped, the ketosteroids rose. The patient was subsequently re-treated with *o,p′*DDD, and again there was a drop in ketosteroids.



Text-figure 4.—Study of a patient with adrenal carcinoma treated with ortho, para prime isomer of diphenyldichloroethane (*o,p′*DDD).

Throughout this treatment period, the multiple pulmonary metastases increased, and the patient died from respiratory insufficiency. From a review of her X rays, we would not have been able to say that any change in tumor growth had been accomplished. However, the changes in ketosteroids did inform us that there had been some effect. Subsequent studies with *o,p′*DDD have demonstrated objective regression of disease.

Text-figure 5 is a similar instance of the same phenomenon. Along the abscissa are plotted the gonadotropin titers of this patient with metastatic trophoblastic disease. This was the first patient treated with methotrexate. Initially she received one intravenous injection and then a course of methotrexate. The marked decrease of gonadotropins during this brief period is apparent. It was not until later, however, that there was a measurable change in pulmonary metastases. Thus



TEXT-FIGURE 5.—Study of a patient with metastatic trophoblastic disease treated with methotrexate. Along the abscissa are plotted the gonadotropin titers.

again, only the presence of an indicator of tumor activity allowed the early conclusion that methotrexate affected tumor activity. This subsequently proved to be a measure of tumor growth.

It is fair to say, then, that the production of hormones or any measurable material, such as acid phosphatase, by a tumor provides an index of tumor activity which may be convenient for tumor screen. It is necessary to prove, however, that changes in such an index reflect corresponding changes in tumor growth before this can be used as consistent evidence of response.

Another type of cancer-host relationship is potentially exploitable. I have listed a number of humoral syndromes which have been produced by metastatic cancer:

Carcinoma of the bronchus with inappropriate secretion of antidiuretic hormone
Cushing's syndrome by a number of metastatic carcinomas
Hyperaldosteronism by metastatic carcinoma
Carcinoid syndrome by metastasizing bronchial adenomas
Osteolysis without bony metastases, with a variety of carcinomas
Erythremia with hypernephroma and cerebellar hemangioblastoma
Aplastic anemia with thymoma
Hypoglycemia with a number of large retroperitoneal tumors
Mysthenia gravis with thymic tumors.

These are some of the more obvious clinical or humoral syndromes produced by tumor or metastatic cancer. It is easy to visualize that there may be more subtle metabolic changes produced by some cancers which we have not been able to utilize as yet. It is, of course, incumbent upon the investigator who treats a patient with one of these tumors and notes a change in, say, the hyperinsulinism or the excretion of calcium, to prove to his colleagues and to his own satisfaction that this reflects a change in tumor growth and not merely some facet of the multiple metabolic activities of the tumor. However, these represent potentially exploitable situations, and in this field of metabolic activities of tumor and the production of compounds by tumor, one may be able to derive experimental situations which will prove valuable in the screening of chemotherapeutic agents.

Dr. Zubrod: Another problem which was referred to repeatedly is this: When do you stop a study; when do you know that you have enough patients to make an estimate of what you are doing? For example, consider the problem in the surgical adjuvant study. At the end of the study it was quite apparent that the patients who had received the alkylating agents had a higher mortality rate than the controls. Could the study have been stopped earlier? Could we have recognized this difference earlier by a different experimental design? I think the sequential design technique referred to in several papers is one possible solution to the moral problem of discontinuing a study as soon as a significant difference appears. Dr. Edmund Gehan will comment on sequential analysis.

Dr. Gehan: The best way to design a trial is evident only after the trial has been run. So, in planning a trial, it is difficult to say which particular design is best.

With this in mind, I would like to discuss the sequential analysis of clinical trials, which is appealing, since everyone would like to have the opportunity to look at results for consecutive patients and use that information to decide whether to stop the trial or continue. A sequential trial is not always the most appropriate, since each trial must be considered on its own merits, but I would like to indicate some of the advantages and disadvantages of a sequential trial.

The first advantage, which Dr. Zubrod mentioned, is that a sequential trial gives a valid answer to the following medical problem: If, during the course of a trial, it becomes evident that one treatment is superior to the other, sufficient to convince the investigators that the difference is real, then they are faced with the ethical question of whether or not to withhold what they believe to be a superior treatment from half the patients under their care. A sequential analysis provides a stopping rule so that the trial is halted when a difference occurs between treatments such that the superior drug can be accepted with small chance that it is not superior. This chance should be specified in advance. As an aside, if we do not have a valid stopping rule and we stop the trial, then we might be recommending the indiscriminate use of an agent before it is established as superior. This would be just as unethical as not stopping early enough.

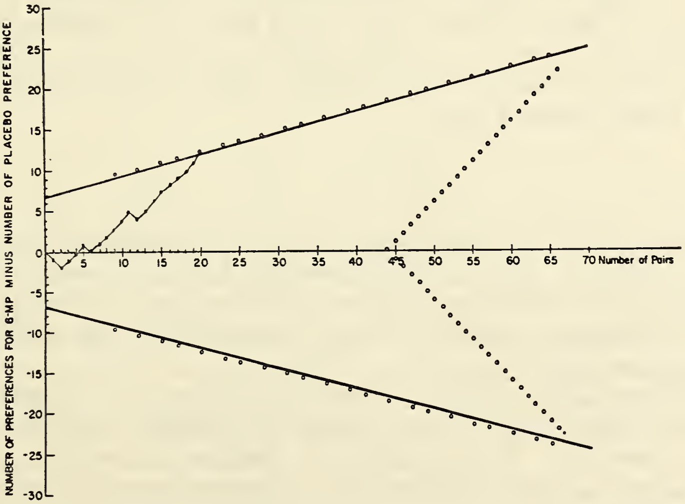
Another advantage of a sequential trial is that the average sample size is smaller than for a nonsequential trial.

One of the disadvantages of running a sequential trial is that a particular end point must be specified, such as percentage of responses or duration of responses. This single criterion would have to be spelled out in advance. In some cases, we might not wish to settle on a single criterion, but we would like to look at, say, percentage of responses and survival time before deciding to stop the trial.

Second, since the idea of a sequential trial is to decide whether one agent is superior to another, it does not lend itself readily to providing an estimate of the difference between agents. Although we may have a valid stopping rule, we may have a poor estimate of the real difference between these agents.

The sequential trial, which the leukemia B group is using in a remission maintenance study, was aimed at finding new agents in the treatment of acute leukemia by testing them in previously untreated patients. We are going about this by using an old agent of known effectiveness, 6-MP, and testing it against a placebo to decide which can maintain a remission longer.

Text-figure 6 presents the setup for this particular trial. All patients going on study are treated for 1 month with steroids. If they go into remission, they are begun on the sequential phase of the trial. The patients in remission—and these are all children under 20 years of age—are paired randomly between 6-MP and placebo, and the length of each remission is recorded. If a longer remission is noted for 6-MP than for placebo in a particular pair, then a preference is for 6-MP. The text-figure presents the boundary lines that provide valid stopping rules for the given number of pairs of patients. A feature of this trial is that the maximum number of pairs of patients is 66 and the minimum number is 9 before a valid stop is possible. If the true preference rate for 6-MP is 75 percent, then we would be 95 percent certain that the sample path



Text-figure 6.—Restricted sequential procedure applied to preferences designed to be sensitive to proportion of 6-MP preferences equal to 0.75.

would go out on the upper boundary. Here, 6-MP is declared the superior agent. Similarly, if the preference rate for placebo is 75 percent, we would be 95 percent certain that the sample path would go out on the lower boundary. The decision would then be that placebo is superior. If both agents were equally effective, with a resulting preference rate of 50 percent for each, we would be 95 percent certain that the trial would continue to one of the boundaries on the right. The conclusion would then be drawn that neither agent has a preference rate 25 percent superior to the other.

The dotted line is simply an example. It does not represent anything that has happened on this particular trial, since results have not begun to come in yet. The sample path shows that the preferences in the first 2 pairs of patients were for placebo, but that then there was a run of preferences for 6-MP, eventually ending in a preference for 6-MP. If the trial is completed in this fashion, we would be able to stop after 19 pairs of patients and be 95 percent certain that 6-MP is really superior.

Dr. Zubrod: The final problem that the panel wishes to bring to your attention is: Are these highly designed trials necessary? Could we do just as well by continuing to collect data in the manner in which individual physicians think is appropriate? I think it is clear from several of the things that have been given here at the conference that this is not true. The surgical adjuvant studies are an example in which one could not have made an estimate of the danger of this procedure without the design it entailed. There are dozens of other examples which Dr. Sidney Farber will summarize in his address. In closing, I want to call on Dr. George Escher who is a valued member of our Experimental Design Committee and a representative of the Sloan-Kettering Institute. Perhaps by virtue of asking Dr. Escher to close, the panel may pay tribute to the late Dr. Rhoads who made so many contributions to the chemotherapy of cancer and whom we miss so much. Dr. George Escher will reiterate some of the findings of the new knowledge that was gained from cooperative studies in breast cancer which perhaps could not have been learned without highly designed trials.

Dr. Escher: The cooperative breast study was the product of a carefully designed protocol. This protocol benefited from the errors of earlier cooperative and independent studies as well as from the trends of the objective regressions obtained. These earlier studies also raised more questions than they answered.

By far the great majority of mammary carcinoma in the female is the infiltrating duct variety. There is, however, distinct variability in how it will metastasize. The distribution of dominant spread was analyzed in 438 patients, and survival from onset of therapy was calculated without regard to response.

Table 1 details this information and shows how poor the prognosis is with a visceral dominant lesion; *i.e.*, 83 percent 1-year mortality as compared with 43 percent with osseous dominant and 27 percent with local soft tissue dominance.

TABLE 1.—Advanced mammary carcinoma—steroid hormone therapy influence of dominant lesion on survival from onset of therapy

Dominant site	Incidence		One-year mortality	
	Number	Percent	Number	Percent
Visceral (liver, lung, brain)	218	50	182	83
Osseous	158	36	67	42
Soft tissue (breast, skin, lymph nodes)	62	14	17	27
Total	438	100	266	61

Because questions were raised about the variable responsivity of these patients relative to their postmenopausal status and to androgens versus estrogens in the greater-than-10-year postmenopausal patients, further stratification was added to the design so that 12 groups were formed.

Table 2 represents the consolidated data from the cooperative breast group. It demonstrates that both the dominant lesion and the postmenopausal status can have a profound and significant effect on the rate of regression.

TABLE 2.—Testosterone propionate: percentage—objective regressions

Dominant lesions	Years postmenopausal				Total percent
	<1	2–5	6–10	>10	
Local	6	17	27	43	30
Osseous	13	18	21	23	20
Visceral	4	7	14	20	14
Total percent	8	14	20	23	20

It is obvious from these 2 tables that a study could easily be set up with patients more than 10 years postmenopausal, presenting only osseous or local soft-tissue dominant lesions, treat them with an active androgen, and obtain a higher objective response rate and longer survival when compared to the results with testosterone propionate used in all 12 groups.

Similarly, one could easily demonstrate a 40 to 50 percent objective regression to hormone manipulation. Patients who showed regression after castration are the best candidates for further objective regression. Selection of such patients would seriously bias the evaluation of an androgen, adrenalectomy, or hyphophysectomy. If patient choice is further restricted to those with osseous or local soft tissue metastases, an even higher response rate is possible.

In conclusion, all the variables of disease behavior must be taken into account in designing an experiment.

Dr. Zubrod: This closes the presentation of our panel on new techniques and problems. I do not suppose the techniques are really new and I do not think that we have solved any of these problems. We have tried to discuss some of the difficulties in the study of drugs in cancer in man. One of the pleasant results of this conference is that there are physicians and scientists who are so reasonable and of such good will that they will sit down together and try to find answers to these many diseases that we lump together under the term cancer.

The other heartening thing about the cooperative studies has been the realization that the findings from many of these studies have already been translated into the improved care of the cancer patient.

The Future of the Cancer Chemotherapy National Program [1]

SIDNEY FARBER, *Children's Cancer Research Foundation,* and *Harvard Medical School, Boston, Massachusetts*

I HAVE a choice between two courses: to read a paper not yet prepared for this occasion on the future of the cancer chemotherapy program or simply to talk informally about some of my impressions during these two magnificant days. I have chosen the second.

This is a heartwarming occasion, all the more so because what you have heard and participated in represents an accomplishment of only 3½ years of organization. What you have witnessed would never have been possible if there had not been created a truly national program with international ramifications, set up with the aid of sponsoring groups interested in the field of cancer, with which you are so familiar through the offices of a center located at the National Cancer Institute in Bethesda. It would not have been possible if there had not been a devoted group of people in chemistry, biology, pharmacology, and the various aspects of experimental pathology and clinical medicine, who were willing to put countless hours of time and energy into meeting on many long and weary Washington days when most of them would have liked to have been home at work. If these people had not created through their voluntary cooperation a series of baselines on which we could build, we could not have screening and chemicals. The vast number of antibiotics would not be ours if industry had not been brought voluntarily into this program. Nothing that you have heard in the clinical sessions would have been possible without the kind of agreement that Dr. Gordon Zubrod brought out in his closing remarks.

I will begin by expressing our appreciation to Dr. Stuart Sessoms and to his staff for arranging the conference. He has a devoted staff to serve his hard-working panels and committees that cooperated to give you the conclusions that you have heard.

I am not going to say too much about the future of this program, because today, while a historic occasion, is just a few months early in the series of events which Dr. Sessoms and his staff have planned. Various committees have been working on a mass evaluation of the entire cancer chemotherapy program. The groups of people working in chemistry, pharmacology, biology, screening, and the clinical panels under Dr. Ravdin have been asked by Dr. Sessoms to work with him and his staff in evaluating critically everything that has been done and in planning constructively for the future. A report will be available soon, and at that time we will have the opportunity, through Dr. Sessoms, Dr. Heller, or Dr. Shannon, to give this new plan of activity to all concerned with this program.

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[1] Presented at Conference on Experimental Clinical Cancer Chemotherapy, Washington, D.C., November 11, 1959.

This planning is something we do not fear but for which we are ready. Only 4½ years ago, when this program began, there was a period of hard work, anxiety, and concern over the use of new conceptions in research in the field of cancer. This marks the largest voluntary cooperative effort in the history of medical research in this country. It was quite clear that such an effort would be guided by groups, and the guidance, as you know, has come directly from a series of committees made up of citizens from all parts of the country, with the aid of men in other countries. This has been a voluntary effort on a completely democratic basis.

But when this was started, the word *directed* program was used in the congressional mandate. I am going to borrow here a statement by Dr. Warren Weaver, of the Sloan Foundation, on the subject of directed research. He quotes the usual definition as found in Webster and then he states:

> "The fact is that many persons use the phrase, directed research, with no overtones whatsoever of autocratic authority and simply to mean research that is directed towards or aimed or focused upon some recognizable purpose. Often, this may be a noble purpose of great scientific and social significance. Such a purpose may act as a large and wholly proper incentive and to serve the purpose effectively it may prove necessary to equip the program in question with the kind and amount of planning and even of central guidance which would not be necessary, useful, or congenial in the case of other types, usually much more individual types of research.
>
> "The National Cancer Chemotherapy program is a good instance of a program which is directed toward a recognizable goal and a good example of a program which by its nature requires a considerable degree of central planning and of coordination of its component parts. Certain parts of this program, admittedly, involve a large amount of somewhat routine and repetitive work. [All of you who have served so devotedly in this program know exactly what those words mean.]
>
> "Certain parts involve the practical business of setting up facilities, such as those necessary to assure sufficient supply of experimental animals. Certain parts require, for obvious scientific reasons, a high degree of uniformity of procedure so that experiments done in various places by various people can be usefully combined. In order that persons of the highest qualification will be glad to work within such a program, two things are essential. First, it must be clear that the planning of the program be carried out by scientists of unquestioned competence and of really relevant experience and under circumstances that assure that these scientists have taken the necessary time and trouble to deal seriously with the situation.
>
> "Second, it must be clear that the rules impressed are not permanently and stupidly frozen but that they will be re-examined at suitable intervals, both by the advisory experts and by those working within the program, to see if they require change."

We owe a debt of gratitude to Dr. Weaver for stating this so clearly. This program, of necessity, from the beginning has combined whatever people mean when they use the terms "basic" and "applied" research. I would join those who have spoken on the program in passing lightly over any past controversy on whether this program is stifling basic research in encouraging applied research or whether much more basic research is required.

I would like to state categorically that anyone in this country or in other countries who has had a rational plan or approach, or a basic approach to the field of cancer in any one of its aspects, has not lacked support—certainly has not lacked a hearing. If there are better ideas than those presented they are eagerly sought, and they will be gratefully received by any of the many examining bodies which pass on applications for support of research in the National Cancer Institute, American Cancer Society, and foundations of any of the other voluntary associations in the country concerned with cancer.

Under Dr. Heller, first with Dr. Endicott and now Dr. Sessoms, this program has grown enormously in these few years. Is it large enough? This question I hope we will be able to answer before we conclude this meeting. Dr. Sessoms and his staff have listed the accomplishments of this program in the various panels and areas covered by the Cancer Chemotherapy National Service Center. This is an extraordinary document, *see* page 303, and a record of truly great accomplishments in many fields outside of the actual care of the patient or the search for chemical agents. The by-products alone have been worth the price of the program so far.

In the clinical program we could not have been more fortunate than to have the leadership of Dr. Ravdin. Because of his spark, enthusiasm, and his great organizing ability, those working closely with him and those on the many subcommittees of the clinical panel were able to provide guide lines which, even though still not complete, brought effectively together the work of some 200 groups with about 500 different research workers on the clinical side alone.

There have been some criticisms of the way the program has been conducted. If there had not been, I would have been very much concerned for the good of the program. Such criticisms should be encouraged, and those who are not in agreement with the way the program is progressing or the way decisions have been made by the many committees and panels concerned in this large undertaking should come foward, talk with their peers, and make suggestions which will lead to improvements.

Many minor disagreements have been brought up by various clinicians who reported their clinical findings and heard some, too, by the chemists, pharmacologists, and biologists interested in screening. We must remember what Ludwig Pick emphasized many years ago: "Between two opposing opinions there lies not the solution of the problem but the problem itself." Let us look always between two opposing opinions, identify the problem, and then see if we can construct another research program to answer the question so defined.

The many different chemical agents, which have been and are being studied, can be divided into two main groups: those that are already on the shelves of industry or of universities and those that are made for this particular study. Actually, the number of chemicals taken off the shelf and studied in this program is not large. In the main, people select chemicals which are apt to have a specific interest biologically and the number being made is increasing, as Dr. Baker told us. When he speaks of 600 additional compounds of one kind, a biologist, pharmacologist, or clinician will be delighted, but at the same time fearful of the immense amount of labor which one organic chemist can cause for so many different biologically inclined scientists.

It was heartening to hear from Dr. Baker that the alkylating agents stand at the frontier of interest. We have heard a difference of opinion. If we hear that alkylating agents are of no value and hear at the same time from other people that these are just beginning to be of interest, I believe that we can be forgiven if we listen to those who bring sufficient proof to whet our appetite for more alkylating agents.

Dr. Bergel, with whom we have had many delightful discussions on his visit to this country, introduces the possibility, as have other people, that we join agents with two modes of action, and he talks of the possibility of purified antibodies to tissues or organs being attached to alkylating chemicals. This introduces a fine conception which should be brought forth in practical terms to actual compounds that can be studied in man.

As a suggestion of further combinations of known anticancer agents, we might mention antimetabolites and alkylating agents or expansion of the antimetabolite field, which certainly has not been fully explored. There is evidence of the enzyme deletion, for example, Potter's work, and in the area of enzymes and coenzymes, enzyme inhibitors mentioned in a thoughtful discussion by Dr. Bergel recently. With the huge programs of development in chemistry in the field of large molecular polypeptides, we can speak now of the possibility of artificial enzymes. For example, there is the definite possibility, expressed by chemists working in this field, that in time they will be able to create artificial enzymes and perhaps artificial proteins.

In the field of antibiotics, we reach an area where rational approach is impossible. Here, there are hundreds and thousands and perhaps millions of possibilities. We are grateful that industry has taken such an immense interest in this aspect of the problem because only through industry would it be economically possible to have available the tremendous number of antibiotic beers and fermentation products which now are of interest as anticancer agents. That a few such antibiotics are actually in use in man is one of the most encouraging discoveries in this entire field of cancer chemotherapy because here, as Dr. Baker expressed it, with the thinking done by the microbiological agents in the structure of chemical materials, we have great possibilities for use in man. We also have great possibilities for increased work for our biochemical and organic chemical colleagues in the unraveling of what nature has already performed for us.

The hormone analogues have become available only because of the Cancer Chemotherapy Service Center national program. This vast number of hormone analogues made for other purposes in industry would never have been applied to the cancer program in individual institutions in this short time. In the internal control of some forms of cancer lies the answer, very likely, rather than in the destruction of tumors by something that might act in a more crude manner.

Finally, we have a great deal to expect in the growing field of natural products, in plants and plant products. Dr. Murray Shear had one of these important programs. There is now a number of programs in which plant products are being systematically studied, their extracts produced, chemical identification of those which prove to be of interest made, and some of these actually studied in man. The number seems to be almost without limit unless we find one chemical substance which will be common to a great many of these plant products.

In the field of screening, I would like to pay particular tribute to Dr. Murray Shear who had a pioneer program in this country, and to Dr. Chester Stock and his colleagues who began early and with unusual courage and devotion stayed with a large and burdensome program. Men who entered this field early and systematically studied the action of chemical compounds with admittedly imperfect screening methods deserve great credit because, if they had not persisted, we would be years behind the achievements we have reached.

I would like to mention, particularly to the clinicians present, the importance of the mouse, the rat, the microbiological system, or any screening system which shows an effect of a chemical agent on a living organism. In this field the directions are still not clear. It was far less promising 12 to 15 years ago than it is today, since the support of one's colleagues could not be obtained with ease, and those who wanted to work biologically, make chemical compounds, or search in experimental pathology or pharmacology for leads, required some kind of justification for what they were doing. This method of screening, although it was used at first with only microscopic effects by Dr. Shear on Sarcoma 37 and by Dr. Stock on Sarcoma 180, provided something for people to look at. They could take photomicrographs, bring them to a scientific meeting and show their colleagues, and they could persuade their superiors that there was something going on biologically which was of interest.

If we had had any one of the clinically studied compounds mentioned at this meeting, in the mouse or the rat 10 to 15 years ago, I think this program would be many years ahead of our present achievements. Ten to 15 years ago it would have been impossible to have attracted a group of clinicians the size of this audience today who would be willing to work in a field that was so unpromising and that seemed so far removed from the patient. If there is no other justification for screening techniques, this alone is justification, and I would like to support those who continue to fight for the use of any biological screening method which has scientific support for its existence.

It is clear that we may be misled by the screening method. May I take you back a few years to the excitement when 8-guanazolo was the main topic of conversation in cancer circles because it acted on a certain kind of screening agent, the *Tetrahymena*, which, though metabolically somewhat different than man or the mouse or the rat, was metabolically prepared so that this particular chemical approach could come out of such a screening method. In that case, we were perhaps misled momentarily, as far as human cancer was concerned, but the preoccupation with that area of chemistry has yielded many fine results. This, certainly was not wasted.

There was also the fortunate sensitivity of the Walker sarcoma to the alkylating agents. I used to wonder, but not any more, whether the chemists at Chester Beatty, who are working devotedly and intelligently with alkylating agents, really required the Walker tumor to give daily approval for their persistence in this field or whether they did not believe in the validity of their conceptions so that they were willing to continue on and on and make more compounds, some of which formed the backbone of cancer chemotherapy in humans today. The screen there, while not perfect, has served its purpose.

It is quite possible that the criticism of the so-called cure of the transplanted tumor in the mouse is not good criticism. It is true that this is an artificial system. The tumor is transplanted, but we must remember that 10, 12, or 14 years ago we did not have a single agent which would destroy such a tumor in a mouse. When we have evidence that a chemical compound can completely destroy a tumor in a mouse, we have something that is of great biological significance, even it it does not apply immediately to man. We must understand just what we are measuring and what we are looking at when we have such a screening method.

It is quite possible that when all of our research from different parts of the world in the field of screening is compiled, we may be able to do without the human tumor in the hamster, the human tumor cell in tissue culture, the microbial systems of the mouse, or the rat tumors. We may find some actual tissue change or body-fluid change in the experimental animal that we can carry directly to humans after suitable pharmacological study.

This leads me to the field of pharmacology. We have heard discussion with terms that did not exist some time ago, such as quantitative screening. We are interested in excretion, in localization of effect in the metabolism of chemical compounds, mechanism of action, time-dose relationships, and so on. This is a dilemma. We are fortunate that we have men trained in pharmacology by Professor Marshall, and that we have the advantage of his great experience and knowledge in this field.

From my own experience, biologically and clinically, there is no greater need at the present time than for data from men who are expert in this area of pharmacology and experimental pathology. There is greater need for data than for anything else in order to expedite our studies in man. At a recent meeting of a number of scholarly men in this field of pharmacology and biochemistry related to pharmacology, I got the distinct impression, and I sympathize with them up to a point, that they will be ready to do any kind of pharmacological study, once clinicians find that an anticancer agent has some real meaning in man. But they would prefer to reserve their energies until that time so they could study occasional substances and not do large amounts of work that might be wasted later.

I sympathize with them because they have research ideas of their own they want to pursue, but I would hope that they would find it possible to create programs so that this kind of work could go on without interfering with their own original research. This is a matter of major importance. When we treat man with a chemical compound, we have problems such as those brought out in this last panel and all through this conference. It is unfortunate that we still do not know whether to give a compound every hour, four times a day, once a day, once a week, or once a month. What validity is there in the time-dose relationships which have become a matter of tradition for the chemical compounds that are now of standard use against cancer in man?

These problems must be re-examined. We will move more quickly here when some of Dr. Zubrod's remarks can be put into actual practice. Now, we are confronted with two great problems, the solution of which will be of great importance to human beings with cancer.

The first concerns toxicity and the methods of detoxifying an agent so that a patient can be protected against toxicity or can be given something that will counteract toxicity. I refer here to many different types of approach. You are all familiar with those concerned with bone marrow infusions, attempts to detoxify nitrogen mustard or other compounds, or protecting the human being by nutritional pathways.

The second concerns the solution of the problem of resistance. More clinical and biological studies will have to be directed toward the effective use of the anticancer agents we now have.

One of our speakers said that his group did not learn much about new drugs because they did not have many to work with, but they did learn more about the natural history of the disease, the methods of evaluation, and the planning of studies. I would think that those concerned with the clinical panel would consider this remark a great compliment given by someone working in one of these cooperative programs, who could pick out something that really is of essence in this type of group study. These cooperative studies have given us the experience of the mathematician and the statistician in the production of experimental design.

I would make only one comment about the clinical remarks that we have heard in the planning of these studies. The word placebo is used only when we have nothing of real value to give a patient in its place. The clinicians are not depriving a patient of something which might help him or prolong his life simply to give him saline or something without any therapeutic meaning.

What these combined studies here have meant to the patient cannot be measured simply. The fact that some 500 investigators are working clinically and that many hundreds more are stimulated by these 500, has brought a new kind of confidence to the patient with cancer that is incurable by methods of proved value. There is hope based on something which the doctor can convey only when he really knows or believes that he may have something beneficial for this patient tomorrow, next week, next month, or, if the patient can survive that long, next year.

This is the greatest by-product of the entire cancer chemotherapy program for the patient. What will come of all the studies, criteria, methods, planning, and experimental design is secondary in importance because we know that these change as men get experience, as they learn to work together, and as they find that they can develop better methods of investigation.

So far in the clinical program there are direct effects which are of definite value. Only permanent survival or cure, as far as the tumor is concerned, will impress those who are most deeply concerned with the patient. We have not yet reached that stage of cancer chemotherapy clinically, but retinoblastoma, a rare tumor, may be cured, according to those at Columbia University and Presbyterian Hospital who report this, when an alkylating agent is combined with radiotherapy. They may even save the eye; this was described in the Columbia reports as a definitive and curative therapeutic regimen.

In regard to choriocarcinoma, the word cure has been used. I still would not use it because not enough time has gone by, but in a percentage of the females studied, not in all, methotrexate, a chemical compound known for a number of years in the treatment of leukemia and other related tumors, produced regressions at the National Cancer Institute of a kind that were not ever experienced before. Whether this will be permanent—why doesn't this apply to 100 percent of the patients? why doesn't this apply to the male with the same kind of tumor?—constitutes important problems to be solved.

In children there is very definite improvement and an important increase in survival in children with acute leukemia when a number of the agents with which you are so familiar are used.

We are beginning to see long-term improvement, up to 3 years, in children with Wilms' tumor with metastases to the lungs after combined treatment with actinomycin D and radiotherapy. The life saving action, temporarily, of chemical agents, such as nitrogen mustard or ACTH, in lymphosarcoma involving the mediastinum is well known.

You are all familiar with the striking changes in carcinoma of the ovary, in neoplastic effusions, in some patients with widespread melanoma, and now we are beginning to see changes in gastrointestinal tumors. These cannot be documented yet in terms of percentage of improvement or increased survival. The time is too short and these agents, obviously, are not those which can completely kill cancer throughout the entire body when there is widespread neoplastic disease.

But we may view the findings so far either with complete pessimism and point to the failure of chemical agents to provide permanent cures on a large scale, or we may point to these failures as encouraging signs and feel we have arrived somewhere, if, in daily practice in the care of patients with disseminated cancer, we are able to improve even a small percentage for long periods and increase their survival.

As we go along, we must increase our knowledge of methods of clinical evaluation of anticancer drugs. I am not going to discuss any of the biochemical methods which have been mentioned, but obviously here is the field of challenge to those clinical investigators who have available to them the tools of biochemistry, laboratories of microbiology, and other essential laboratory divisions that are part of an expert investigation clinically.

An up-to-date résumé of the effective anticancer agents presently in use in man, with as clear a percentage of effect in survival as can be obtained from the written records and from the work of this group, is on page 307.

In closing, may I say that for the future there will be increased planning—now possible on a better scale than ever before because of the increased amount of data we have available and the basic information that has come from the preclinical sciences as well as from the clinical wards. There will be a search for a perfect screen, if such can be found, so that we can cut down this interminable lag between the discovery of a chemical or an antibiotic, for example, and its use in man. There will be increased rational planning of agents, perhaps with a greater knowledge of the exploitable biochemical differences between the normal cell and the cancer cell. Or perhaps there will be a discovery here of a much more subtle approach to the cancer cell than the present sledgehammer agents that we now use for the correction of those biochemical defects in the cancer cell which make for cancer.

It is very likely that there will be a union of the field of virus and cancer, on one hand, and chemotherapy, on the other, with prevention or control either endocrinologically or chemically. There is more than a suspicion that these viruses, which may be activators of cancer, act as chemical substances rather than in the sense that we use the term in the field of infectious disease.

This may take the form of looking anew at the cancer problem and putting less stress on the swelling or the tumor part of the cancer, less stress on the chemistry of the tumor cell or the cancer cell, less stress on the appearance and the site and pathological effects of tumor, and much more on the underlying disease which is either the cause of cancer or for the state of affairs which we call cancer. The tumor may be an unessential part of the cancer picture. It may be merely an expression of the basic change. It may be no more important causally than the cavity found in tuberculosis. And in tuberculosis, we must remember that the problem is not only the tubercle bacillus, but something within the host, too, which makes it possible for the red bacillus to cause its damage.

There must be greatly increased preclinical drug evaluation and pharmacology. The increase in the number of clinical groups and the number of participants will be of greatest importance. That increase should begin now, just as rapidly as resources can be obtained to support the work of good minds in good institutions in which people are as yet unable to work because they do not have the necessary support.

In order to carry out effectively this program and to give the kind of support to the clinicians and to the participants in all parts of this program, more devoted people of fine scientific training will have to be recruited to help Dr. Sessoms and his staff in the splendid work which they are doing. Finally, there will have to be improved techniques of recording the results of research as we go along and progress in the evaluation of anticancer agents in man so there can be rapid communication, much more rapid than is now true, between and among the research workers in this country, and in all countries of the world.

Let us hasten the day of the realization of our goal. From the Chinese, may I leave with you the admonition, "Then do good and disappear." Once we finish our job, it is my hope that you will join those who have gone before to the great opportunities for chemotherapy in the fields of mental health and mental disease. Here, new chemical pathways are beckoning and the great goal of mental peace for the world is at stake. There could be no greater challenge to those who one day will be responsible for the conquest of cancer.

Indirect Benefits of the Cancer Chemotherapy Program

The cancer chemotherapy program of the National Cancer Institute was created under congressional mandate to discover effective substances for the treatment of cancer. Two major approaches are used by the Institute to achieve this goal: a coordinated program developed on the advice of panels composed of experts in all disciplines relative to the cancer problem, and grant support for research by our country's top scientists who develop their own research ideas and request support for them.

During the 4 years the program has been operating, the largest screening facility in the country has been organized and at present some 50,000 materials are screened each year; 17 clinical study groups have been formed and are currently evaluating 110 compounds in 200 institutions; existing chemotherapy programs have been implemented, and new programs initiated. In addition, through increased grant support and a new mechanism for support of research, the contract, a nationwide coordinated program of hundreds of interrelated research projects are being carried on in universities, hospitals, government laboratories, and private industry.

Although the program has contained some imbalances due to its rapid growth, a sound basic structure has been built on a framework of cooperating scientists and organizations throughout the country. The past 4 years have been spent in developing the basic structure, adequate policies, and research procedures, as well as learning to do cooperative cancer research on a large scale. The most important phase of the program is yet to come—when the vast potential of outstanding scientist, funds, and facilities can be realized in the form of actual discoveries.

As the chemotherapy program has developed, a number of indirect benefits have become evident. Perhaps the two most important are: (1) the program has encouraged more scientists to study the problem of cancer than would have done so in the normal course of events, and (2) a more universal definition of cancer problems, approaches, and therapy has been made. Increased knowledge from this program has emphasized the need for additional studies on many biological, biochemical, and biophysical processes occurring in both healthy and cancerous cells. Such new information has also suggested different approaches to the study of cancer, and has contributed significantly to other fields of science.

Additional indirect benefits have been realized. The program has:

Facilitated the training of hundreds of scientists and clinicians not only in cancer research but in the basic sciences through employment on Cancer Chemotherapy National Service Center (CCNSC) grant- and contract-sponsored work as well as through training grants.

Developed the first cost-type research contract program in the Department of Health, Education, and Welfare (DHEW), which now comprises approximately 110 contracts with academic institutions, nonprofit research organizations, and the pharmaceutical industry.

Gained the confidence and active collaboration of the pharmaceutical industry through amplification of the DHEW patent policy, thereby stimulating industrial interest in the cancer problem and indirectly increasing the productive capacity of industry.

Contributed to fundamental chemistry through development of new or unusual methods of chemical synthesis.

Stimulated synthesis of unusual chemicals and production of drugs of unusual types for clinical investigation in large quantities unavailable through other sources.

Supplied steroids, rare chemicals, and intermediates, including labeled compounds, to investigators not able to obtain them from other sources.

Developed organizational arrangements to provide large numbers of mice and rats with known genetic background.

Aided in the development of mammalian genetic centers in academic institutions to promote teaching and training in mammalian genetics.

Provided financial support for the maintenance and preservation of important genetic strains of mice and rats.

Developed specifications for minimum standards for mouse production.

Established diagnostic laboratories for detecting and controlling ectromelia, salmonellosis, and other diseases of laboratory animals. These facilities are available to all mouse breeders and users.

Supported intensive investigation of tissue culture, microbiological systems, various tumor systems, and human tumors grown in heterologous hosts so that studies can now be initiated to see if they will indicate compounds useful against human tumors more accurately and at less expense than do present-day mouse tumor methods.

Established extensive facilities for preclinical pharmacology and toxicity testing.

Made new steroids available for clinical trials, for assay and for experimental tumor work; brought many other steroids, which were rare and commercially unobtainable, back into scientific circulation, making them available to research workers for the first time in many years.

Provided for rapid characterization, by assay, toxicology, and tumor testing, of large numbers of compounds. This involved setting up the largest hormone assay program ever attempted; in the course of this a great deal was learned about the statistical evaluation and standardization of assays, and new specifications and new assays were developed.

Made it possible for small academic groups to have their new compounds assayed without expense to them. This has been a great source of encouragement to chemists without local biological services or industrial support, and especially to young investigators and graduate students who have not yet had a chance to prove themselves.

Stimulated interest in the physiological aspects of the tumor-host relationship through attempts to find tumors of endocrine significance, thus broadening the scope of the attack on cancer.

Organized cooperative clinical studies to yield quantitative data in a shorter time than any single institution could, and to develop the best therapeutic measures for specific types of cancer.

Stimulated the interest and cooperation of many clinicians in the care and drug management of cancer patients, which has led to better care of patients with advanced cancer.

Enlisted the cooperation of the Veterans Administration in cancer trials, which has made available additional clinical resources for research and education and has also provided better medical care for more veterans with cancer.

Organized a national standardized system for reporting end results in cancer to give essential baselines on the effect of treatment of cancer in man from over 50,000 new cancer cases per year.

Sponsored an international group to compare cancer end results in the United States, Great Britain, France, Norway, Denmark, and Finland.

Developed biometric units or departments in several medical schools and other institutions to provide statistical consultation for cancer chemotherapy and other biomedical research.

Provided guidance and assistance in statistical aspects of experimental design in laboratory and clinical research.

Developed mechanical processing of biological-chemical data on a larger scale than was ever before attempted.

Made available the first truly unselected, complete data on a national basis of operative complications and mortality from gastric, pulmonary, and rectal resection.

Established a large abstracting service on the cancer literature.

Sponsored six conferences to interest scientists in cancer chemotherapy and to bring together scientists of all disciplines to hear papers and exchange ideas. Approximately 1,000 scientists attended those conferences at which 165 scientific papers were delivered. Proceedings of three of the conferences will be published.

Published screening data, bibliographies, literature surveys, and *Cancer Chemotherapy Reports* to aid and stimulate research.

Stimulated interest in both cancer chemotherapy and basic science through lectures by CCNSC staff and panel members to both technical and lay audiences.

Stimulated the publication of scientific papers by supporting research grants, from which came 150 articles in 1958 alone, and by supporting research contracts which resulted in 90 papers since the beginning of the contract program.

Stimulated a large number of grant applications for basic research by bringing more scientists into contact with chemotherapy.

Set the pattern for other research problems which are best approached by extensive, interinstitutional, cooperative efforts.

Chemotherapeutic Agents of Confirmed or Potential Value in Human Cancer

Drug	Primary or Illustrative Area of Disease Wherein Activity is Manifested
6-Mercaptopurine	Acute leukemia
5-Fluorouracil	? Breast carcinoma
ACTH	Acute leukemia
Hydrocortisone	Acute leukemia
6-Chloropurine	Acute leukemia
Amethopterin	Acute leukemia; choriocarcinoma
Chlorambucil	Chronic lymphocytic leukemia; ovarian carcinoma
Myleran	Chronic granulocytic leukemia
TEM	Lymphomas
Nitrogen mustard	Hodgkin's disease
Thio-TEPA	Lymphomas
Urethan	? Multiple myeloma
Prednisone	Acute leukemia
Cortisone	Acute leukemia
Prednisolone	Acute leukemia
Cytoxan	Lymphomas
Actinomycin D	Wilms' tumor
Thioguanine	Acute leukemia
? Testololactone, 1-dehydro	Breast carcinoma
Diethylstilbestrol	Prostatic carcinoma
Testosterone propionate	Breast carcinoma
? 2-α-Methyl-4,5-α-dihydrotestosterone	Breast carcinoma

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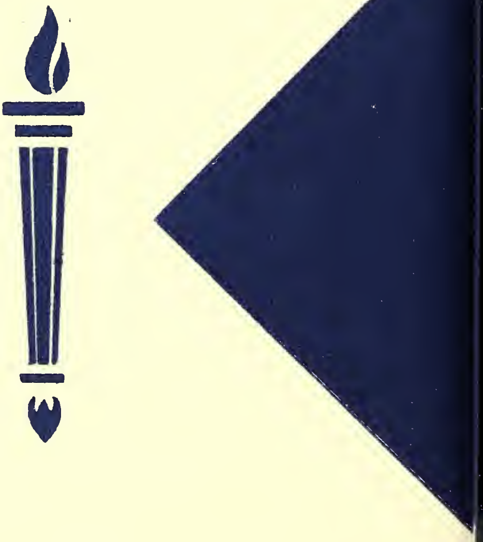

NATIONAL CANCER INSTITUTE MONOGRAPH NO. 4

Symposia
Tumor Viruses

September 1960

U.S. DEPARTMENT OF HEALTH, EDUCATION & WELFARE

Public Health Service

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Cancer Symposium

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Symposium

Phenomena of the Tumor Viruses

Sponsored by
Virology and Rickettsiology Study Section
National Institutes of Health, Bethesda, Maryland
Held March 25 and 26, 1960
New York, New York

Edited by
Joseph W. Beard

Introductory Remarks

Dr. Joseph W. Beard, Duke University, Durham, North Carolina

It has been but a few years ago that the problems of the virus-induced tumors attracted the attention of only small numbers of investigators in this country and abroad. Furthermore, the work was greatly restricted in scope with respect both to the material available for examination and to the limitations of the methods of study. Since 1950, however, the possibilities for investigation of the virus tumors have undergone a most remarkable and previously totally unforeseen expansion. The attainment of the present status of the problem has been related to a number of factors, principal among which have been the discovery of additional virus tumors and, fully as important, the development of new techniques and their application to the problem. Another major factor has been a sharp increase in the support of work in this field. As the result of these influences, the number of investigators now actively concerned with the study of virus tumors has increased almost explosively.

It has become evident that a simple approach to the many problems of the virus tumors can no longer be expected to yield comprehensive results. Instead, it can already be seen that productivity is dependent, in the main, on the application of the various, and, in many instances, highly specialized techniques which include, in addition to the older classical methods of virology, the more complex aspects of immunology, tissue culture, cytochemistry, electron microscopy, and, finally, biochemistry at the molecular level.

Because of the complexities created by the significant advances in widely separated areas of research, a critical problem at the moment is the rapid correlation and dissemination of the knowledge derived with the various disciplines. It has been the purpose of the Virology and Rickettsiology Study Section of the U.S. Public Health Service to hold this symposium to provide a forum common to all workers in this country interested and intimately participating in virus tumor research. By this means it is hoped to increase significantly the general understanding of the problems, the findings, and the expectation of progress in these studies.

It should be mentioned that much of the stimulation of the recent tremendous growth of the field of virus tumor research has been related to the efforts of Dr. J. E. Smadel and Dr. Carl Baker, who have been personally instrumental in fostering expansion of the work. Dr. Ralph Meader, together with Dr. Harvey Scudder and Dr. Robert Backus, has been very generous with his time and interest in promoting the administration of the program. The program committee of the symposium is Dr. Howard B. Andervont, Dr. W. Ray Bryan, Dr. Hilary Koprowski, and Dr. J. W. Beard. Acknowledgement should be made to Mrs. Courtenay Harrison for preparation of the preprints and to Mrs. Dorothy Beard and Miss Marie Purdy for their compilation of name lists and the distribution of preprints.

Finally, it is distressing to realize that two of the people who were to have participated in this meeting will not be here: Dr. Charles Oberling and Dr. George Edwards, who died in just the last few weeks. You all must know a great deal of Charles Oberling, who was the director of one of the most productive laboratories in the world in this field and who made a lifetime work of stimulating research on virus tumors. He will be represented at this symposium by Dr. Françoise Haguenau from his laboratory.

Dr. Edwards had not been in the field so long, but he was a man of great promise, tremendous enthusiasm, and outstanding ability in his work.

Acknowledgment

This symposium was supported by research grant C-4986 from the National Cancer Institute, National Institutes of Health, Public Health Service, to Dr. Joseph W. Beard, Duke University, Durham, North Carolina.

Tumor Virus Research Program [1]

JOSEPH E. SMADEL, *M.D., National Institutes of Health,[2] Bethesda, Maryland*

THE basic question before this conference is: What etiologic role, if any, do viruses play in the malignant neoplasms affecting the human race? Despite almost a half century of study of tumors in lower animals caused by agents with the classical characteristics of viruses, the exact biological mechanisms by which these viruses induce malignancy in cells remain undetermined. Certainly it is worthwhile to continue to investigate viral-tumor model systems in animals until the mechanism of carcinogenesis is illuminated. However, the virologist need not delay his etiologic approach to human cancer while waiting for the biologist to solve carcinogenesis.

During the past few years, there has been developing a ferment of interest in viruses and human neoplasms. There are many reasons for the current activity: Tissue-culture techniques have opened a wider vista for sighting hitherto unrecognized viruses than did the previous expansions of the horizon which accompanied the introduction of the suckling mouse, the embryonated egg, and the ordinary laboratory mouse into common isolation procedures. Morphologic techniques, as exemplified by electron microscopy of thin sections, have given tantalizing results with human tumors, while immunofluorescent microscopy and the more recent ferritin antibody procedures for use in electron microscopy (*1*) seem to provide potent weapons for studies on viruses and cancer. Immunologic procedures have become more delicately sensitive. The scientific world has been stimulated by the infectivity of naked viral nucleic acids. Smallpox and measles, in which infection is almost invariably associated with a typical full-blown clinical and pathological picture, are no longer regarded as the prototypes of most viral diseases. Instead, poliomyelitis, with its high incidence of subclinical infection and rare paralytic manifestations, is considered typical of a large number of viral diseases. Moreover, salivary-gland viruses and adenoviruses have joined the group exemplified by herpes virus and other microbial agents which persist for long periods in clinically recovered persons.

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[1] Presented at a Symposium on Phenomena of the Tumor Viruses, New York, N.Y., March 25 and 26, 1960.

[2] Public Health Service, U.S. Department of Health, Education, and Welfare.

Translation of such findings to the virus-cancer problem provides support for two ideas: (1) Tumor development may occur in only a small proportion of people who are infected with the hypothetical inciting virus, and (2) the causal agent may persist in the individual for years, eliciting its carcinogenic effect long after initial infection. Another development that has stimulated virologists is the findings with the polyoma agent. These demonstrated, more dramatically than earlier observations with chicken malignancies, that multiple types of tumors may arise as a result of infection with a single virus. Can we hope for a simplification in man such as this in mice and chickens?

While virologists have been persuaded to join the exploration of the viral etiology of human cancers primarily because of one or another of the scientific developments just mentioned, there were other factors which assisted in their conversion. A number of investigators were concerned, during and immediately after World War II and the Korean War, with virologic diseases in foreign lands of importance to Americans stationed there and to the indigenous peoples. Others were engaged in work which culminated in the successful control measures for poliomyelitis. Many such persons were willing to rededicate their efforts to another difficult and important cause, *i.e.*, cancer. Fortunately, the interest of the lay public, reflected through the action of Congress, has made funds available for support of research in this field.

Like most of you, I view the problem of the viral etiology of human cancer with hope and enthusiasm. Moreover, I have confidence that investigators entering this field will attain the success here that they have had in their research on the classical viral infections of man.

Although my assigned task was to present briefly certain of the background information which has excited the interest of virologists, particularly, in working on the problem of the viral etiology of human cancer, I cannot refrain from discussing diagnostic virology, which has for many years provided a central theme for my own work. Most cancer investigators have had little exposure to the subject, hence they are not likely to be impressed with the need for the cautious attitude and the stepwise process of elimination that have become ingrained in the virologists who sort out the etiologic agents of human infection. The principles of diagnostic virology have been learned slowly and painfully over the last half century (*2*). I hope that cancer workers will use this knowledge to advance rapidly in their own field; it would be regrettable if they fumbled, even for a short time, while rediscovering the information and principles.

The basis of diagnostic virology is to be found in Koch's postulates as revised by Rivers in 1937 (*3*). The latter's ideas, restated somewhat in the light of experience with a host of subsequently recognized viruses, are essentially as follows: A virus is presumed to be of etiologic significance in a given human disease if (1) the agent is present in the tissues or excretions of most persons in the acute stage of the illness and is absent or infrequently present in normal persons or patients with other diseases, and (2) patients who yield the virus during their acute illness develop with regularity, during convalescence, specific antibodies against the agent. Virologists have practically abandoned the idea that Koch's final postulate, *i.e.*, reproducing the disease in a normal host, must be fulfilled in man if a virus is presumed to be a human pathogen. However, an increasing number of the viruses which have been shown by the criteria of Rivers to be of etiologic importance in nonfatal human disease have been inoculated into human beings. Sometimes such transmission studies have resulted in the reproduction of the classical manifestation of the original disease, *viz.*, dengue, measles, and trachoma. In other instances the experimental disease has occasionally mimicked the original but more frequently has reproduced only part of the clinical picture, *viz.*, lymphocytic choriomeningitis and adenovirus infections. Finally, some laboratory strains of viruses, notably the attenuated polioviruses, induce only subclinical infection.

One would hope that a thorough application of old, new, and yet-to-be-developed isolation techniques would yield viral agents from human cancers with which one might fulfill Rivers' criteria. Encouragement will certainly be given to those who try such approaches. However, along with encouragement should go a word of caution, since these approaches will undoubtedly yield numerous agents that will be unrelated etiologically to human cancer, even though they may have been present in the patient and not indigenous to the systems used for the isolation work. The persistent efforts over the years to propagate in the laboratory the viruses of hepatitis and of epidemic hemorrhagic fever provide documentation of the frequency with which agents may be recovered that are not of etiologic significance in the diseases under study. Application of the serologic diagnostic criteria mentioned revealed the irrelevancy of the extraneous isolates; in brief, either the patients developed no antibody against the isolate or if antibodies were present they did not increase in titer during the disease.

If the near future fails to provide techniques by which a viral agent presumably causing human cancer can be put through the standard procedures to fulfill the diagnostic criteria demanded as proof of etiology, then we might decide *a priori* what data would partially satisfy the basic demands of diagnostic virology.

Our first compromise with principle might be a temporary relaxation of the demand for the appearance of, or increase in, antibody during the course of the disease. Since cancer generally is of insidious onset and has been present for some time before being recognized by the physician, one would anticipate that any antibody response to the hypothetical virus would already have approached its maximum before the patient is examined. With this relaxation, the criteria would then be (1) consistent recovery of the transmissible agent from tumors of a given type along with absence of, or low incidence of, the agent in patients with other diseases, and (2) consistent presence of specific antibody in patients with the tumor. Consistency is the key word here. The ubiquitous viruses which many persons carry for appreciable periods of time, such as herpes simplex, salivary-gland virus, and members of the adenovirus group, would be irregularly distributed among the isolates. For any of the known carrier type viruses to be of even transient interest in the subject under discussion, it would be necessary to demonstrate a high degree of positive association between it and a given tumor.

One might postulate a series of compromises, each of which would retreat a little further from the established methodology and principles applicable to the diagnosis of acute viral infections. However, it would save time to go immediately to that point beyond which only chaos would exist. The minimum evidence for the presumptive establishment of a viral agent as being causally related to human cancer might be fulfilled without propagating the agent in the laboratory and without demonstrating specific antibody by one of the common test-tube techniques. In such an approach, the procedures of electron microscopy might be substituted for those of isolation and transmission in animals, and fluorescent antibody techniques might replace the older serologic methods.

Earlier I spoke of the tantalizing results obtained by electron microscopic examination of human malignant tissues. Intracellular particles resembling those of certain of the established viruses have been observed in some tumors after much searching, and also in occasional control materials. Investigators have been discouraged by the irregular occurrence and sparsity of such particles in cancers of man. Moreover, they have been justified in their cautious interpretation of the observations. In order for the findings to contribute to the partial fulfillment of minimum requirements, they must be demonstrable with great frequency. This obvious statement, in the face of the laborious efforts which have gone into obtaining the presently available data, is no doubt irritating to those investigators who contributed to the subject. The intention is not to provoke but rather to encourage. I recall the difficulty in finding rickettsiae, which are rather large structures, during electron microscopic examination of thin sections of infected yolk-sac tissue that was known to contain at least 10^9 infectious organisms per gram and to contain innumerable rickettsiae when stained smears were examined by ordinary microscopy. The problems encountered in studying small particles in tumor cells might be expected to be infinitely more difficult. The significance of viruslike submicroscopic intracellular particles would be measurably increased if it could be shown that the patient with tumors possessed specific antibody which reacted with them. Perhaps Singer's ferritin-labeled antibodies (*1*) prepared from serum of the patient might be applied to thin sections of the patient's tumor and examined by electron microscopy.

The fluorescent antibody techniques have proved so valuable in so many fields of endeavor that they will undoubtedly find increasing application in the studies on cancer. One would hope that cells with masses of the particles occasionally encountered by electron microscopy would fluoresce specifically in the presence of labeled antibodies from the patient.

It goes without saying that each sample of tumor tissue used for either electron microscopic or immunofluorescent studies of the general type discussed should be tested by standard virologic isolation techniques to detect the presence of ordinary human pathogens, particularly the viruses, which commonly induce the carrier state. These, if present, might be detected and shown to have induced antibody in the host by the microscopic techniques mentioned, but the standard diagnostic procedures would provide more efficient means for recognizing them.

In closing, I would like to read the final sentences from Rivers' address in 1937 on "Viruses and Koch's Postulates" (*3*):

> "Changes, notably the more extensive use of tissue culture techniques and serologic reactions, will in the future undoubtedly occur in the methods of establishing the specific relation of viruses to disease; the number of changes will be limited only by the amount of ingenuity of investigators. To obtain the best results, however, this ingenuity must be tempered by the priceless attributes of common sense, proper training, and sound reasoning."

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Properties of a Leukemia Virus [1]

JOHN B. MOLONEY, *Laboratory of Biology, National Cancer Institute,*[2] *Bethesda, Maryland*

RÉSUMÉ OF STUDIES IN THE MURINE LEUKEMIA VIRUS FIELD

The viral etiology of the avian leukosis complex was established in 1908 by the work of Ellerman and Bang (*1*). However, it was not until over 40 years later that a similar relationship of viruses to certain of the murine leukemias was demonstrated.

Gross Leukemia Virus

In 1951 Gross (*2*) showed that acellular filtrates of the hematopoietic organs of AK mice, which had developed leukemia spontaneously, would induce the disease in mice inoculated shortly after birth.

One of the "fundamental features" of these studies was the use of mice less than 16 hours old as test animals. The results obtained with older mice were erratic, the incidence of leukemia low, and the latent periods greatly extended.

Under similar experimental conditions Gross (*3*) and Stewart (*4, 5*) later showed that the leukemic tissue filtrates would also produce tumors of the parotid gland and subcutaneous fibromyxosarcomas. Filtrates prepared from any one of these neoplasms reproduced all three tumor types upon reinoculation into susceptible hosts.

In 1957 Gross (*6*) reported the development of a highly active "Passage A strain" of leukemia virus. Originally derived from spontaneous AK leukemia, the agent was passed serially in newborn C3H mice. This passage material produced tumors in a greater percentage of inoculated mice in a shorter period than the original strain of the virus. Of particular interest is the additional finding by Gross (*7*) that the "Passage A" virus produced leukemia but not parotid-gland tumors in suckling and young adult C3H mice.

The recovery and cell-free passage of an X-ray activated leukemia agent in Bittner C3H mice has also been described by Gross (*8–10*). The relationship of the "Passage X" virus to the "Passage A" agent remains to be determined.

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[1] Presented at a Symposium on Phenomena of the Tumor Viruses, New York, N.Y., March 25 and 26, 1960.

[2] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

Physical data on the Gross virus indicate that it is inactivated at 65° to 68° C. and that it remains stable at −70° C. as well as under the conditions of lyophilization. A particle size of 50 to 70 mμ, determined by sedimentation and electron microscope observations, has been reported by Gross (*11*). Dmochowski and Grey (*12–14*) and Bernhard and Gross (*15*) described the presence of particles in the virus-induced leukemic tissues and in extracts of these tissues. Although these particles varied in size and structure, the predominant elements, according to Bernhard *et al.*, were of *Type A* ("doughnut"-shaped particles) with a diameter of 75 mμ.

Immunological studies (*10*) with "Passage A" and "Passage X" leukemia filtrates have established that these materials elicit a neutralizing antibody response in a heterologous (rabbit) host system. The presence of hemagglutinins in heat-inactivated "Passage A" leukemic tissue filtrates has also been described (*16*). The mouse erythrocyte agglutinin could be separated from the active principle of the filtrates by centrifugation, which left open to question the direct relationship of the agglutinin to the tumor virus.

The histologic characteristics of the viral-induced leukemia were similar to those of spontaneous lymphocytic leukemia and to those of the lymphoid neoplasms induced by total-body X irradiation. McEndy *et al.* (*17*), Furth (*18*), and Law (*19*) have demonstrated that removal of the thymus gland decreases the incidence of spontaneous leukemia in normally high leukemic strains of mice. Kaplan (*20*) showed that thymectomy had an inhibitory effect on the incidence of radiation-induced lymphomas in C57BL mice. In addition, Gross (*21*) reported that thymectomy either prior to or after inoculation of "Passage A" leukemic filtrates inhibited the viral induction of lymphocytic leukemia in C3H mice.

Schwartz Leukemia Viruses

In 1957 Schoolman, Spurrier, Schwartz, and Szanto (*22*) described the recovery of a lymphoid leukemia-inducing agent from the brain tissue of a leukemic Swiss mouse. The virus produces generalized lymphocytic leukemia in their substrain of adult Swiss mice. Regardless of the route of inoculation, intraperitoneal or subcutaneous, the latent period was 1 to 3 weeks after virus inoculation.

Of particular interest is the observation that the virus could be recovered not from the lymphoid tumor but from the brain tissue of the infected mice. This fact and the unusually short latent period of the agent distinguish this virus in the mouse leukemia field. The agent is inactivated by heat (30 min. at 56° C.) and by formalin.

The gross pathologic findings, which differed from those of the AKR leukemia, were significant splenomegaly and hepatomegaly and frequent thymus enlargement; however, peripheral lymph-node involvement was slight. The outstanding observation was a massive involvement of the mesentery, which often extended to the urogenital organs.

A more recent report by Schwartz *et al.* (*23*) described the recovery of a similar leukemia-inducing agent from the brain of a C3HeB mouse with a spontaneous lymphoblastic leukemia. This agent has the same characteristics with regard to thermolability and filterability as those described for the Swiss mouse agent. The C3H agent is effective in adult mice of various C3H sublines and produces leukemia at a high incidence. The latent period is relatively short, usually not exceeding 46 days for the most resistant animals.

Friend Leukemia Virus

In 1957 Friend (*24*) isolated a leukemia virus from the spleen of a Swiss mouse that had been inoculated, shortly after birth, with a cell-free preparation of Ehrlich carcinoma. The Friend virus is apparently strain specific. Adult mice of the Swiss and DBA/2 strains are highly susceptible, whereas mice of the PRI, C57BL/6, A, C3H, and (C58 × BALB/c) F_1 strains are nonreactive.

The "leukemia-like" disease produced by the virus has been described by Friend (*25*) and further interpreted by Furth and Metcalf (*26*) as a reticulum-cell disease. It is associated with nonmalignant erythroblastosis, without involvement of the lymphoid or myeloid systems. The disease is characterized macroscopically by marked hepatosplenomegaly. The latent period in susceptible adult mice is relatively short, the spleen becoming infiltrated and palpable within 2 weeks after the intravenous inoculation of virus.

The leukemic nature of this virus disease has been questioned on the basis of the inability of the neoplastic cells to produce solid tumors. However, recently Friend and Haddad (*27*) and Buffett (*28*) have reported the production of localized tumors from virus-induced leukemic spleen and liver implants. Buffett and Furth (*29*), using similar tissues, described the development of a transplantable reticulum-cell sarcoma in Swiss mice.

Physical data on the mouse leukemia virus of Friend show that it is stable for extended periods when stored at −70° C. and when dried in the frozen state. It is inactivated in 30 minutes at 56° C. and when treated with formalin (1:200) or ether.

Electron microscope studies by de Harven and Friend (*30*) on pelletized leukemic tissue filtrates and on leukemic spleen, liver, and bone marrow sections have revealed the structure of the virus particles. The observed particles were of types A or C, according to the classification of Bernhard and Guérin (*31*), and were found to have an average diameter of 78 mμ. Similar results were obtained by Dalton *et al.* (*32*) in their electron microscope studies of a solid tumor. This latter neoplasm originated from a transplant of virus-induced leukemic spleen cells that had been maintained in tissue culture for varying periods.

The antigenic nature of the virus has been described by Friend (*33*). Homologous and heterologous antiserums produced in mice and rabbits, respectively, were found specifically to neutralize infectivity of the leukemia agent. Additional studies showed that serums from mice with leukemias induced by either the Gross or the Schwartz agent failed to neutralize the Friend virus, which indicated no direct immunological relationship between these serums and the leukemia agent of Friend.

Graffi Leukemia Virus

Graffi in 1957 (*34*) summarized extensive studies on a myeloid leukemia virus recovered from five transplantable mouse tumors, including Sarcoma 37. This virus inoculated into newborn mice of strains Agnes-Bluhm, sg (sooty yellow) and db (dilute brown), produced granulocytic leukemia in a large percentage of the recipients. A high proportion of the induced leukemias were of the chloroleukemia type. Adult mice were found to be sensitive to the agent, but to a lesser degree. However, fractionated total-body X irradiation increased the incidence of the disease in older animals.

A lack of strict species specificity of this virus is indicated by the report of Graffi and Gimmy (*35*), describing the induction of "intrathoracic leukoses" in newborn rats inoculated with the agent. In a similar manner cell-free preparations of the rat leukemic tissues retained leukemogenic activity when they were reinoculated into the mouse (*36*).

The myeloid leukemia agent is characterized by the following physical and chemical properties: It is inactivated by heating (65° C. for 30 min.), by lyophilization, and by treatment with deoxycholate, concentrated crystallized trypsin, formalin, and ether (20 min. at 0° C.). It is resistant to treatment with the nucleases, lipase, weak trypsin solutions, and 50 percent glycerin. It is stable within the *p*H range 4.5 to 9.5.

High-speed sediments of the tumor filtrates are reported to contain 10 percent ribonucleic acid (RNA) and to possess a strong adenosinetriphosphatase (ATPase) activity. Myeloid leukemia could be induced by "nucleoprotein fractions" prepared from the filtrates by precipitation at *p*H 4.5 with acetate buffer. In addition, Bielka and Graffi (*37*) have shown that RNA extracted from the myeloid leukemia tissues also produced the disease. An incidence of 10.3 percent was obtained when the ribonuclease (RNase) sensitive extract was inoculated into newborn Agnes-Bluhm mice.

Electron microscope observations by Graffi (*38*) on high-speed sediments of the tumor filtrates and on sections of virus-induced leukemic tissues revealed virus particles measuring 70 mμ and possessing a distinct inner structure of 30 mμ in diameter.

The specific antigenic nature of the Graffi agent is evidenced by the neutralization of the leukemogenic factor with heterologous (rabbit) immune serum. Rabbit antiserum against normal tissues failed to in-activate the virus, and attempts to produce a homologous antiserum have thus far been unsuccessful.

It is interesting to note that, in contrast to lymphocytic leukemia, the spleen, rather than the thymus, plays a crucial role in the histogenesis of the virus-induced myeloid leukemia. Thus Fey and Graffi (*39*) found that splenectomy of the very young animal had a strong inhibiting effect on the development of the leukemia, regardless of whether it was performed just prior to, or several days after, virus inoculation. On the other hand, if splenectomy was delayed 1 to 2 months after virus injection, no inhibitory effect was evident.

Kaplan Leukemia Agent

Kaplan (*40*) established an additional link between certain of the external carcinogens and the leukemia-inducing viruses. He observed that cell-free preparations from a radiation-induced tumor produced the same neoplasm when inoculated into nonirradiated susceptible hosts.

In this respect Lieberman and Kaplan (*41*) recently demonstrated the presence of a leukemogenic agent in lymphoid tumors induced in C57BL/Ka mice by fractionated total-body X irradiation. The recovered agent induces lymphocytic tumors when it is inoculated into nonirradiated newborn isologous and compatible F_1 hybrid mice. After serial passage in these animals the virus produced lymphoid neoplasms in 69 percent of the recipients with a median latent period of approximately 28 weeks.

STUDIES ON A MURINE LYMPHOID LEUKEMIA VIRUS RECOVERED FROM SARCOMA 37

Recent studies in this laboratory have been directed toward a more complete understanding of the nature of a lymphoid leukemia virus isolated from the mouse neoplasm, Sarcoma 37 (*42*, *43*).

This virus produces a generalized lymphocytic neoplasm in 100 percent of the recipients within a relatively short period when inoculated into mice of various ages and strains and into rats. The disease produced is similar to that which occurs spontaneously in certain inbred strains of mice.

Biological Properties

Source of Leukemia Virus

Sarcoma 37 was transplanted from strain A/LN mice [3] to BALB/c mice. It was carried in this inbred line for three tumor generations before the tests were carried out which revealed the presence of a virus.

Sarcoma 37 is maintained as a routine passage series in BALB/c mice and still yields the virus after 50 transplant generations. Such tumor material can be used as a source of the leukemia agent. Additional sources of the virus employed in the studies to be described were: (1) leukemic lymph nodes, thymus glands, spleens, and livers from mice which had received cell-free concentrates of Sarcoma 37 and (2) leukemic hematopoietic organs from mice which had received whole-cell intraperitoneal implants of leukemic tissues, originally derived from cell-free concentrates of Sarcoma 37. The virus-induced leukemia is freely transplantable in adult mice within a given strain. One hundred percent of the recipients develop generalized lymphocytic leukemia in 1 to 2 weeks after intraperitoneal inoculation of the neoplastic cells.

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[3] Mice of strain A/LN bearing Sarcoma 37 were received from Dr. M. K. Barrett of the National Cancer Institute. The sarcoma had been carried in this strain of mice for 195 transplant generations. No leukemias had been observed by Barrett in the tumor-bearing animals.

Recovery of Virus From Source Tissues

The techniques used for the recovery and concentration of the leukemia virus from the source tissues have been illustrated and described in detail in a report published earlier (*43*). In summary, they involve the processing of source tumor tissue by two different procedures, after preliminary extraction and clarification by the following method: A 10 percent homogenate of tumor tissue in 0.15 M potassium citrate was treated with hyaluronidase. The homogenate was then cleared of nuclei and cell fragments by low-speed centrifugation. The supernatant (fraction S1) recovered from this run was further fractionated by either of the following procedures:

Procedure A.—The particulate fraction containing the virus was sedimented from the recovered supernatant (S1), which had been clarified 2 times, by centrifugation at 30,000 × g for 1 hour. The pellets were resuspended in 0.05 M *p*H 6.8 sodium citrate buffer and clarified. The supernatant was recovered and the pellets again washed and clarified. The supernatant resulting from the last centrifugation was pooled with the previous one. The final product was a concentrated particulate fraction containing the biologically active virus.

Procedure B.—Fraction S1 was cleared of large aggregates of cellular material by passage through a Selas XFF filter. The filtrate was diluted and refiltered through an 02 Selas bacteriological candle, shown to be impervious to *Escherichia coli*. To concentrate the virus, the 02 filtrate was then centrifuged at 30,000 × g for 1 hour. The pelletized material was resuspended in 0.05 M *p*H 6.8 sodium citrate buffer, to give the final preparation.

Yield of Virus From Leukemic Tissues

The quantity of virus recovered from neoplastic tissues is dependent in part on: (1) the concentration of the agent in the source tissues and (2) the techniques used to extract the virus.

To determine which of the three most grossly affected organs, *i.e.*, liver, spleen, and lymph nodes, would yield the greatest amount of leukemia virus, a series of 3 experiments was carried out. Leukemic livers, spleens, and lymph nodes (peripheral and mesenteric) were removed and pooled separately from BALB/c mice approximately 8 weeks after inoculation of the leukemia virus. The tissues were tested as individual sources of the leukemia agent. The method of virus extraction and recovery was that described in procedure A. The resulting particulate fractions were diluted in serial tenfold steps in 0.05 M pH 6.8 sodium citrate buffer containing 1.0 mg. percent of hyaluronidase. BALB/c mice less than 36 hours of age were inoculated subcutaneously with 0.1 ml. of the respective dilutions.

Analysis of the biological response data (percentage incidence and time-to-death with leukemia) suggested a relatively low concentration of the virus in the liver tissues. In addition there was a greater variability of the individual animal responses within the dose groups. No difference in the quantity of extractable virus between spleens and lymph nodes was indicated by the data.

Results of the experiment with leukemic lymph nodes as virus source material are presented in table 1. From the high percentage incidence (column 3) and the low average latent period (column 4) values, it is seen that these tissues serve as excellent source materials of the leukemia virus. Further, it is indicated that the procedure used to extract the virus is of value in the recovery and concentration of the biologically active principle from the source tissues.

TABLE 1.—Titration of virus concentrate prepared from leukemic lymph nodes

Log dilution	Number positive / Number inoculated	Incidence (*percent*)	Average latent period* (*months*)
10^{0}	19/19	100	2. 9
10^{-1}	46/46	100	3. 0
10^{-2}	43/43	100	3. 4
10^{-3}	37/37	100	4. 0
10^{-4}	39/39	100	4. 5

*Average time-to-death with leukemia after virus inoculation. All inoculated mice died of leukemia by the 7th month.

Routes of Inoculation

The leukemia virus is effective in mice when administered by any one of the following routes: subcutaneous, intracerebral, intraperitoneal, intravenous, or oral. Regardless of the route of inoculation, the neoplasm produced is always of the generalized type. No tumor appears at the site of inoculation. In weanling and adult mice, the intravenous route is the most effective. On the average, mice inoculated intravenously die with leukemia 1 month earlier than those receiving virus by the other routes. Of interest is the finding that the virus will produce the disease when administered orally. As pointed out by Maduros *et al.* (*44*), in studies on the oral induction of leukemia with the Schwartz mouse leukemia virus, it creates a problem to be considered in quantitative response studies when mice are caged together and when cannibalism occurs.

Host Specificity

As discussed in a previous section, certain murine leukemia viruses are specific with respect to age, strain, and species of the host. It was considered of interest, therefore, to study the influence of these factors on infection by the leukemia virus recovered from Sarcoma 37.

Age.—The susceptibility of BALB/c mice in various age groups to virus-induced leukemia was examined in a series of 4 experiments. In experiment 1 (table 2) the virus source material was Sarcoma 37; in experiments 2 to 4 (table 2) it was leukemic tissue induced by whole-cell inoculation. The agent was extracted from these tissues according to procedure B and in the case of Sarcoma 37, concentrated to 0.59 or 0.75 gm. equivalents per ml.[4] The concentration of virus material derived from the leukemic tissues was either 0.25 or 0.5 gm. equivalents per ml.

Table 2.—Susceptibility of BALB/c mice, at various ages, to virus-induced leukemia

Experiment No.	Virus source	Age at inoculation (*days*)	Number positive / Number inoculated*	Incidence (*percent*)	Average latent period† (*months*)
1	Sarcoma 37	1–16	88/88	100	8. 4
2	Leukemic tissue	31	5/5	100	4. 5
3	Leukemic tissue	42–67	65/69‡	94. 2	4. 4
4	Leukemic tissue	121–246	114/120‡	95. 0	6. 2

*Number inoculated and surviving to weaning age where applicable.
†Average time-to-death with leukemia after virus inoculation.
‡Remaining mice died without tumor during observation period; cause of death unknown.

Mice[5] less than 30 days old received 0.1 ml. of extract subcutaneously, whereas the older mice were inoculated intraperitoneally with 0.2 ml. of virus concentrate.

As shown in column 3 (table 2), the mice in the combined experiments ranged in age from less than 1 through 246 days at the time of inoculation. In each experiment the mice were approximately equally distributed at 1- or 2-day intervals throughout the designated age bracket.

The percentage incidence data (column 5) indicate that death with leukemia was essentially 100 percent in experiments 1 through 4. It is evident, therefore, that the virus is capable of eliciting leukemia in mice inoculated as late as 8 months of age.

Strain.—Although inbred BALB/c mice are used as test animals in most studies carried out in this laboratory, 8 additional strains and 5 F_1 hybrid groups of mice were tested for susceptibility to the leukemia virus. Mice were inoculated at less than 1 day, at 4, or 6 days of age. A 0.1 ml. inoculum containing 0.26 to 0.5 gm. equivalent per ml. virus concentrate was employed. The inbred strains tested were: BALB/c (reference strain), C3H, C3Hf, I, A/LN, DBA/2, RIII, C57BL, and general purpose Swiss mice. The following hybrid groups of mice were also inoculated:

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[4] A 1 gm. equivalent per ml. concentrate is equal to 1.0 ml. of final suspension for every gm. of tissue processed.

[5] All mice used in these studies and the strain specificity studies were from the inbred colonies of Dr. H. B. Andervont, National Cancer Institute.

(BALB/c × C3H)F_1, (BALB/c × RIII)F_1, (BALB/c × I)F_1, (BALB/c × C57BL)F_1, and (BALB/c × DBA/2)F_1. The incidence of the viral-induced leukemia among the hybrid animals was 100 percent, with an average latent period of 3.3 months. With the exception of the C57BL mice, death with leukemia was also 100 percent in all inbred strains inoculated.[6]

The mean time-to-death with leukemia, when considered on the basis of age of the host within a given strain, did not vary significantly from the mean time-to-death of the reference (BALB/c) strain. The leukemia virus is therefore not highly strain specific. However, a low incidence of leukemia obtained for the C57BL mice (2 of 7 mice positive) might indicate a degree of resistance of this mouse strain. This possibility is now being investigated.

Species.—In view of the apparent lack of strain specificity of this leukemia virus, it was considered of interest to extend the host studies to include animals of other species (*45*).

Sprague-Dawley rats [7] less than 36 hours of age were inoculated with the agent derived from a pool of virus-induced *mouse* leukemic thymus glands and lymph nodes. The extract was prepared by differential centrifugation alone, *i.e.*, according to procedure A, and the final product, concentrated to 0.5 gm. equivalents per ml., was suspended in either 0.05 M *p*H 6.8 sodium citrate buffer or 0.12 M sodium chloride solution. The rats received the virus intracranially, intraperitoneally, or subcutaneously. The sodium chloride suspensions were used for the intracranial inoculations, and both citrate and sodium chloride suspensions were employed for the intraperitoneal and subcutaneous routes of administration.

The results of the experiments 16 weeks after virus inoculation are summarized in table 3. From the incidence data (column 4) and the mean time-to-death figures (column 5) it is apparent that the virus, originally derived from mouse leukemic tissues, is effective in producing the same disease in rats at a high incidence level after a short period of latency.[8]

The recovery of virus from the induced rat leukemic tissues has been demonstrated by the passage of cell-free extracts of this material in newborn rats and also in mice. Although the first rat "selective virus passage" experiment is still in progress, both rats and mice are dying with leukemia 7 weeks after inoculation of the rat-derived virus preparation. The leukemia agent, therefore, has not acquired host specificity after a single passage in the rat.

It is of interest to note that the virus-induced rat leukemia is also freely transplantable within the strain of rat. The neoplastic cells, when inoculated subcutaneously in adult Sprague-Dawley rats, produce a localized leukemia or lymphosarcoma at the site of implantation.

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[6] This incidence value excludes mice which died of unknown causes during the observation period.

[7] Furnished by the Animal Production Unit of the National Institutes of Health.

[8] A comparative assay of a 10^{-1} dilution of the same virus preparation in newborn BALB/c mice produced leukemia in 100 pei cent of the recipients, with a mean time-to-death of 15.9 weeks.

TABLE 3.—Virus-induced lymphocytic leukemia in Sprague-Dawley rats*

Route of inoculation	Virus dilution (*log*)	Number positive / number inoculated†	Incidence (*percent*)	Average latent period‡ (*months*)
Intracerebral	0	8/13	61. 0	3. 1
	−1 (0.05 ml.)	16/26	61. 5	3. 5
Intraperitoneal	−1 (0.2 ml.)	21/32	65. 6	3. 5
Subcutaneous	−1 (0.2 ml.)	17/24	70. 8	3. 1

*Results at 16 weeks of experiments still in progress.
†Age of rats at inoculation: <36 hours
‡Average time-to-death with leukemia.

Serial Virus Passage

Attempts to increase the potency of the leukemia virus by repeated passage in newborn mice were undertaken in a group of experiments termed "selective virus-passage series." In principle the techniques were similar to those used to increase the concentration of virus in source tissues in the virus-induced avian neoplasms (*46, 47*). The series was initiated with leukemia virus derived from Sarcoma 37 and processed according to procedure B. Subsequent passages were effected by inoculation of virus derived from the induced leukemic spleens and lymph nodes. Donors of the source tissues were the first animals in each of the respective passages to exhibit macroscopic evidence of leukemia.

The results to date, based on latent period data, indicate that the potency of the virus has been increased with successive passages. The first passage material induced lymphocytic leukemia in 100 percent of the test animals, with an average latent period of 6.4 months. After eight virus passages in newborn mice, the agent now induces the generalized neoplasm in a high percentage of animals as early as 5.5 weeks after inoculation. One hundred percent of the inoculated mice have leukemia within a latent period of 2.5 months.

In this study, latent period is defined as time from virus inoculation to palpable inguinal lymph nodes and/or spleen. Time-to-death after detection of infiltrated organs varies from 11 to 28 days, with a mean of 19 days.

Pathology of the Induced Disease

In mice.—Microscopic examination[9] of diseased tissue sections revealed that the lymphocytic neoplasm was widely dispersed and involved many organs and tissues. No neoplasms other than those of the lymphocytic type have been observed either grossly or microscopically in any test animals. In general, a similar histologic picture was recognized in all the mice examined, whether the disease developed after virus inoculation or after the mice received neoplastic cells. No significant variation was detected in mice of different strains or age groups.

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[9] The author is grateful to Dr. Thelma Dunn of the Laboratory of Pathology, National Cancer Institute, for her review of microscopic slides.

The nucleus of the neoplastic cell was round or slightly indented and it was surrounded by a narrow rim of basophilic cytoplasm. Mitotic figures were frequent. In addition to infiltration of the spleen, liver, and lymph nodes by neoplastic cells, mice with the far-advanced disease also showed infiltration of neoplastic cells in the salivary glands, the kidney, the lungs, and the meninges. It should be emphasized that the histologic material was limited to mice with the fully developed disease, and early stages have not been studied. A more comprehensive morphologic study is now in progress.

Mean cell counts of the cardiac blood from leukemic male BALB/c mice are presented in table 4. These values are compared with those reported by Russell *et al.* (*48*) obtained from animals of the same subline. From the figures given in the table it is evident that the total leukocyte count was elevated. The erythrocyte count was low, and, as a further indication of anemia, the hemoglobin content, determined by the method of Drabkin and Austin (*49*), was also reduced.

TABLE 4.—Cardiac blood cell counts of male BALB/c mice

	White blood count (*mm.*^{3})	Red blood count (*mm.*^{3})	Hematocrit (*percent*)	Hemoglobin (*gm/100 ml. blood*)
Normal*	8.7×10^3	10.1×10^6	46. 5	14. 5
Leukemic	29.1×10^3	7.0×10^6	34. 6	10. 3

*From the data of Russell *et al.* (*48*).

Macroscopic examination of mice with the virus-induced disease presented the usual picture of a generalized lymphocytic neoplasm (fig. 1). Invariably the mice exhibited marked hepatosplenomegaly. The peripheral and mesenteric lymph nodes showed massive involvement. In 90 to 95 percent of the leukemic animals, the thymus gland was greatly enlarged and sometimes obscured the heart on the anterior surface. Mice that developed leukemia after intraperitoneal implantation of neoplastic cells presented the same picture as that described, with the exception that the thymus gland showed relatively little, if any, gross involvement.

In rats.—The pathology of the virus-induced leukemia in the rat is in general similar to that described for mice.

Macroscopic.—Although some rats with the induced disease exhibited the characteristics of a generalized lymphocytic neoplasm that involved the spleen, liver, lymph nodes, and thymus (fig. 2), a high percentage of the inoculated rats showed only marked enlargement of the thymus gland. The average weight of these infiltrated thymus glands was 7.0 gm. (normal thymus, 0.5 gm.).

Microscopic.—Histologic studies on rats with the more generalized disease showed the neoplasm involved many organs and tissues.

The neoplastic cell was generally uniform and of large size in comparison with the normal lymphocyte. It contained a single highly basophilic nucleus and a small rim of nongranulated basophilic cytoplasm (fig. 3). Infiltration by these cells destroyed the normal architecture of the thymus and involved lymph nodes, and extended beyond the boundaries to involve adjacent organs and tissues (fig. 4).

Immunological Studies

The antigenic nature of the leukemia virus recovered from Sarcoma 37 is being investigated. Antiserums produced in rabbits by cell-free virus concentrates have been found to neutralize effectively the leukemia virus. Although the specificity and the absolute titer of the antiserums remain to be determined, preliminary findings indicate that a 1:2 dilution of one antiserum completely inactivated an equal volume of $10^{-1.3}$ dilution of virus. Normal serums had no effect on the leukemogenic activity of similar virus preparations.

Biochemical Properties

The results of preliminary investigation into the biochemical nature of an active virus preparation are shown in table 5. The extract was prepared from a pool of virus-induced leukemic lymph nodes and spleens. The final concentration in terms of gm. equivalents per ml. was 0.5. The total nitrogen figure, 0.462 mg. per ml., when related to biological activity, serves as a measure of the degree of separation of nonactive nitrogenous material from the active agent.

TABLE 5.—Results of chemical analyses of virus extract prepared from leukemic lymph nodes and spleens

Analysis	Mg./ml.
Total nitrogen	0. 462
Total phosphorus	0. 076
Pentose nucleic acid	0. 274
Deoxypentosenucleic	0. 000

The value (0.076 mg./ml.) given for total phosphorus reflects the lipide-, nucleic acid-, and protein-phosphorus content of the particulate suspension.

With regard to the nucleic acid content, no deoxypentosenucleic acid (DNA) was found as measured by the diphenylamine reaction. The absorption of the nitrogenous bases at 2600 Å in perchloric acid extracts of the particulate material was entirely accounted for by the amount of pentose nucleic acid in the acid extract, as measured by the orcinol reaction. These observations strongly indicate that the virus preparation was free of nuclear material and that the agent is an RNA-containing particle.

Physical Properties

Electron Microscope Studies

The ultrastructure of the virus particle and its association with the leukemic tissues are being studied by Dr. A. J. Dalton of the National Cancer Institute. Observations have been made on thin sections of the virus-induced leukemic tissues and on pelletized virus concentrates prepared from these tissues. The techniques used for fixation and staining were similar to those published previously (*50, 51*).

Particles were found in greatest number in an extracellular position in lymph nodes and the thymus glands. The majority of these particles were not well defined; both the nucleoid and outer membranes appeared shrunken and distorted (fig. 5). Occasionally particles were found in the cytoplasm, usually within vacuoles which possessed discrete membranes (figs. 6 and 8). Only rarely were individual particles encountered which appeared to be lying free in the cytoplasm, and these consistently exhibited good internal structure (fig. 7).

Characteristically the extracellular particles possessed a central nucleoid with an average diameter of 65 mμ. The periphery of this nucleoid was more electron dense than its central portion. Two concentric outer membranes were present, the inner with a diameter of approximately 85 mμ, and the outer with a diameter of approximately 100 mμ (fig. 9).

More comprehensive electron microscope studies are now in progress to elucidate further the ultrastructure of the active agent.

Thermal Stability

Sarcoma 37 and the virus-induced leukemic tissues maintain their capacity to produce tumors on implantation and their efficiency as virus source materials when sealed and stored at the temperature of dry ice.

Lyophilization of the particulate materials does not significantly reduce the biological activity of the virus. Data obtained from one experiment indicate no apparent difference in the activity of lyophilized material relative to a frozen aliquot of the same virus preparation.

The leukemia virus prepared in 0.05 M *p*H 6.8 sodium citrate buffer is stable for at least 90 minutes at 37° C. However, the leukemogenic activity of the virus in the same buffer or in 0.12 M NaCl is completely destroyed within 30 minutes when heated at 56° C.

SUMMARY AND CONCLUSIONS

Presented herein are a summary of studies with the known viruses that induce leukemia in murine animals and the results of studies with a virus originally recovered from a connective-tissue neoplasm, Sarcoma 37, but which, when extracted from this tumor, evoked leukemia in other susceptible hosts.

TABLE 6.—Properties of the murine leukemia viruses

Investigator	Origin	Particle size ($m\mu$)	Host sensitivity			Neoplastic cell (*type*)
			Age		Strain	
			Suckling (*percent*)	Adult (*percent*)		
Gross	AK, C58 (spontaneous leukemia)	60–150 (3 types)	62–100	44–60	C3H, C3Hf (Bittner)	Lymphoid
Schoolman and Schwartz	Swiss and C3HeB (spontaneous leukemia)	—	—	15–100	Swiss and C3H	Lymphoid (sarcoma)
Graffi	Sarcoma I, Sarcoma II, SOV 16, Ehrlich carcinoma, Sarcoma 37	70	70	25	Agnes Bluhm, sg, db, (rats)	Myeloid
Friend	Ehrlich carcinoma	78	—	85–100	Swiss, DBA/2	Reticulum cell with erythroblastosis
Kaplan	C57BL/Ka (X-irradiated)	—	69	—	C57BL/Ka, compatible F_1 hybrids	Lymphoid
Moloney	Sarcoma 37	90–110 (extracellular)	100	100	BALB/c, C3H, C3Hf, I, C57BL, DBA/2, RIII, Swiss, A/LN, F_1 hybrids, (rats)	Lymphoid

Table 6 briefly summarizes certain of the biological and physical properties of the virus recovered from Sarcoma 37 and relates these to analagous observations with the other murine leukemia agents.

The present leukemia virus differs from two other agents recovered from solid tumors primarily by the pathology of the resultant neoplasm. It induces a generalized leukemia characterized by a lymphoid type of neoplastic cell. The agent of Graffi induces tumors in mice and rats that are characterized by a myeloid type of neoplastic cell. The Friend virus induces a leukemia in adult Swiss and DBA/2 mice which has been interpreted as a reticulum-cell disease with nonmalignant erythroblastosis.

Among the group of leukemia viruses recovered from spontaneous lymphocytic neoplasms, the Schoolman and Schwartz agent is, as stated earlier, distinguished by (1) the very short latent period for its action, (2) the formation of a lymphosarcoma-like mass involving the mesentery and (3) its recovery from the brains but not from tumors of infected mice.

The Gross agent, the first to be recovered from a naturally occurring lymphocytic leukemia, is apparently highly strain specific; it is most effective in C3H mice of the Bittner substrain. Although the lymphoid neoplasm produced by the virus is similar to the spontaneous disease, fibromyxosarcomas and carcinomas of the parotid gland develop in mice inoculated at birth with the same cell-free preparations. This indicates the presence of more than one agent in the leukemic tissue filtrates. Of significance is the recent development, by Gross, of a highly active virus passage material effective in weanling and young adult C3H mice. Mice older than 1 day at the time of virus inoculation develop leukemia but not parotid-gland tumors.

The direct relationship of the virus described herein to the virus of Gross and other murine leukemia agents must await the outcome of immunological, physical, and biochemical studies. However, as indicated in table 6, the properties which characterize the virus are: (1) Infection is not limited to the very young animal or mice of the same genetic line; (2) lack of strict species specificity is indicated by the viral induction of lymphocytic leukemia in rats; (3) no tumors other than those of the generalized lymphocytic type have been observed, regardless of the strain or age of the test animals; and (4) virus concentrates induce leukemia in 100 percent of the inoculated animals within a relatively short period.

Thus, there is available for laboratory investigations a murine leukemia virus of high biological activity which is not age specific or highly strain or species specific.

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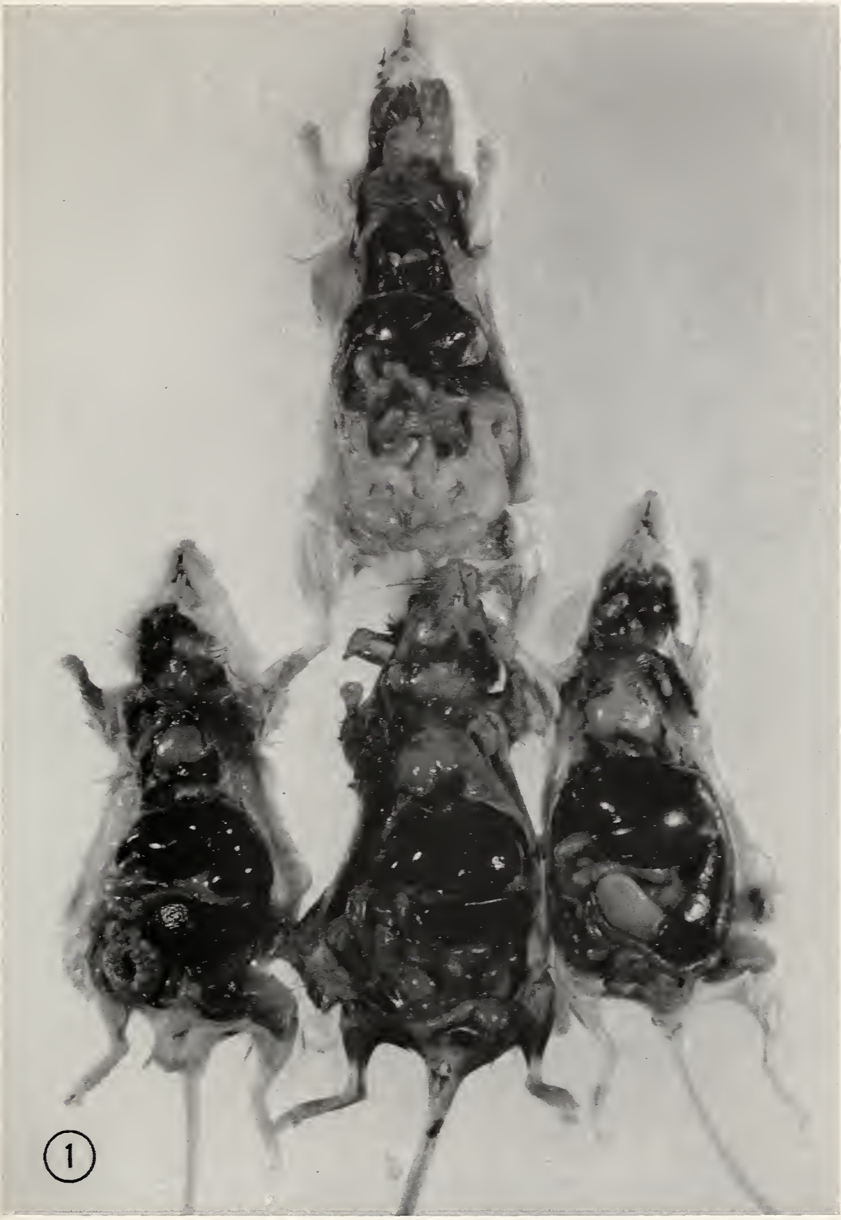
Plate 1

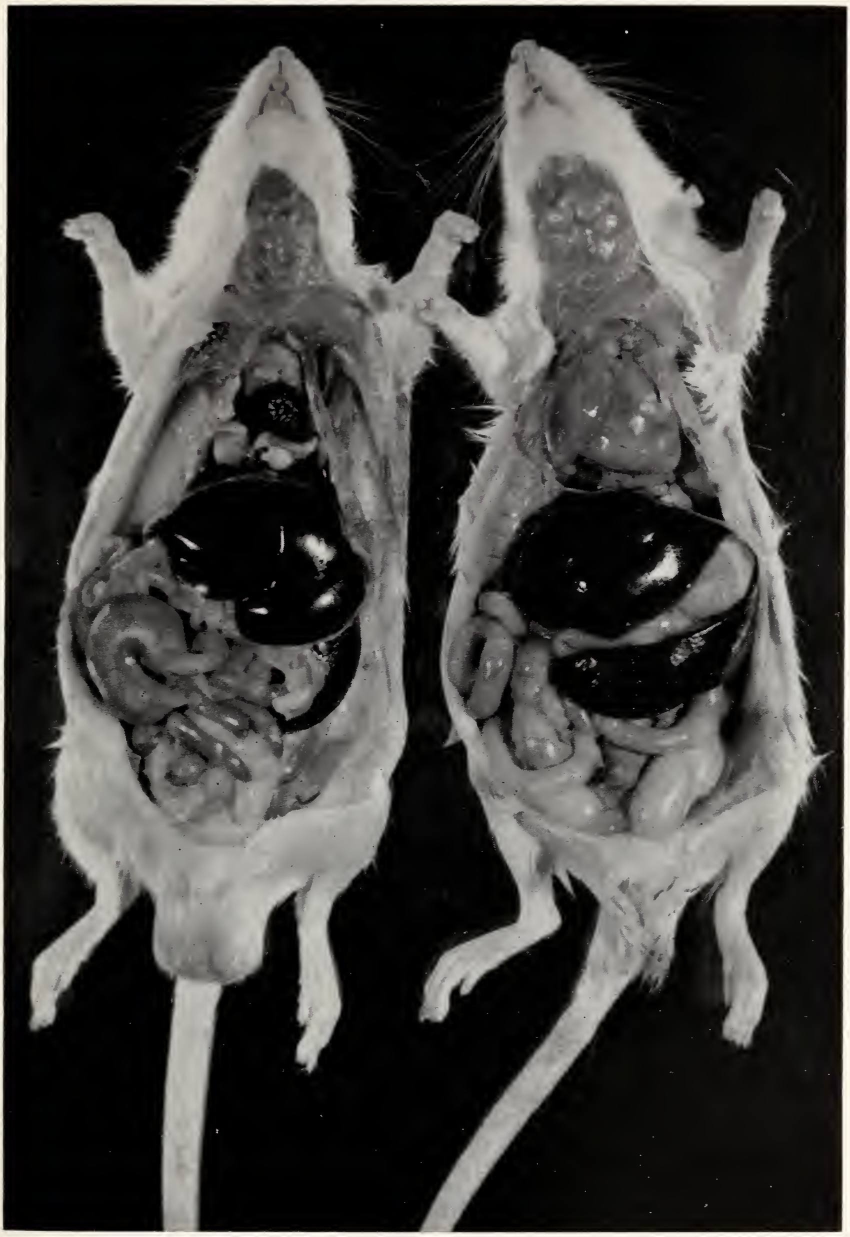
Figure 1.—Leukemic mice of various strains which had received virus shortly after birth. *Above,* normal uninoculated BALB/c mouse. *Lower left,* mouse of strain I. *Center,* mouse of strain C3Hf. *Lower right,* mouse of strain BALB/c. *Note* enlargement of spleen, liver, and thymus gland in all leukemic mice. In addition, *note* enlargement of mesenteric lymph node in inoculated BALB/c mouse.



MOLONEY

Plate 2

Figure 2.—Sprague-Dawley rats. *Left,* normal uninoculated rat. *Right,* rat inoculated with mouse-derived virus shortly after birth and killed at 9 weeks of age. *Note* enlarged spleen and thymus gland in leukemic rat.



MOLONEY

Plate 3

Figure 3.—Sprague-Dawley rat inoculated with mouse-derived virus shortly after birth and killed at 10 weeks of age. Neoplastic cells in tracheobronchial lymph node. *Note* basophilic nuclei and narrow rim of cytoplasm. Hematoxylin and eosin. × 780

Figure 4.—Same rat. Pancreatic lymph node, normal architecture destroyed by infiltration with neoplastic cells that have extended from lymph node to infiltrate adjacent pancreatic tissue. Hematoxylin and eosin. × 150

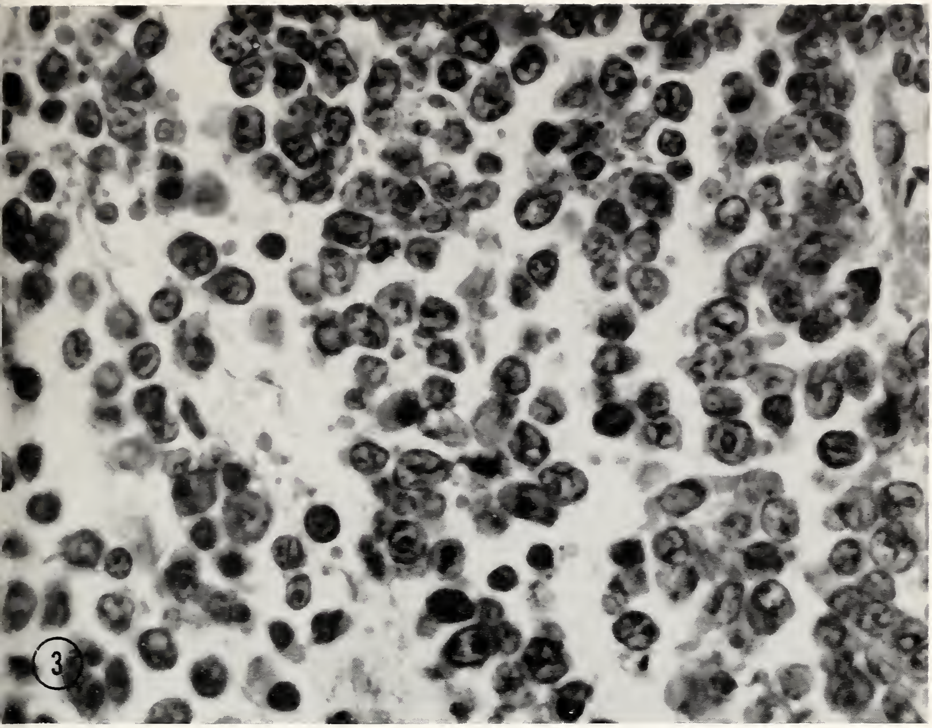

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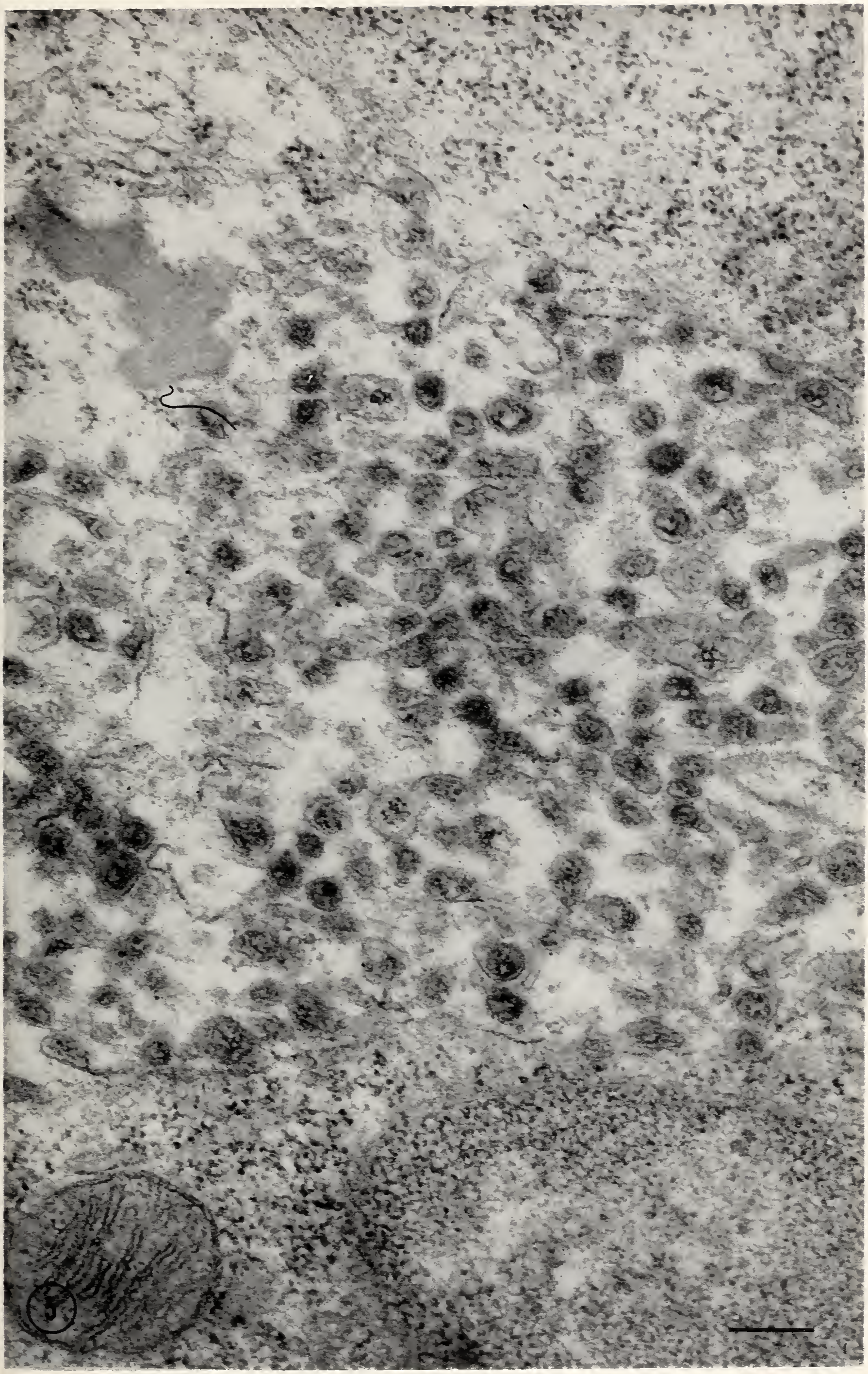
Plate 4

Figure 5.—Electron micrograph of part of section through lymph node of leukemic mouse. Numerous particles varying in size and state of preservation are present in extracellular position. Horizontal line represents 0.1μ. Chrome osmium fixation. Modified lead hydroxide stain. × 70,000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Horizontal line in each figure represents 0.1μ.

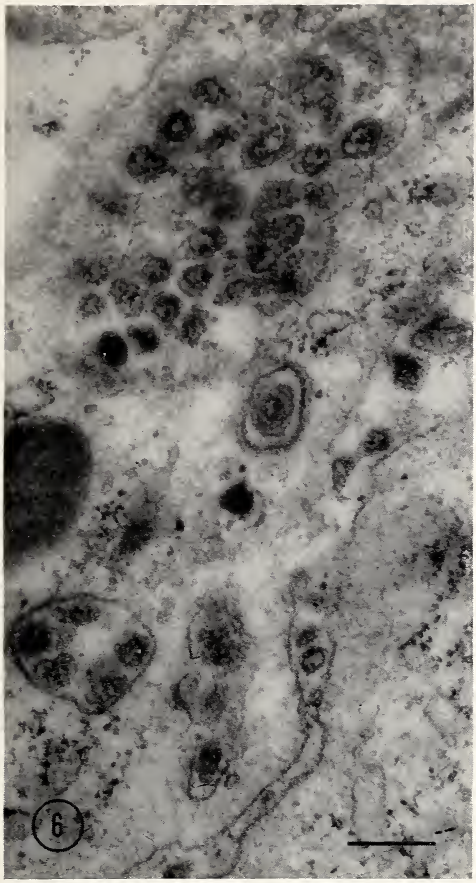
PLATE 5

FIGURE 6.—Electron micrograph of part of cell in mouse leukemic lymph node, showing intracellular particles. Majority of particles are within vacuoles delineated by distinct membrane. A few extracellular particles are present at *right* of figure. Chrome osmium fixation. Modified lead hydroxide stain. × 70,000

FIGURE 7.—From thymus of leukemic mouse. Shows solitary particle (*arrow*) apparently lying free in cytoplasm. This particle appears well preserved. Chrome osmium fixation. Modified lead hydroxide stain. × 70,000

FIGURE 8.—Shows portion of cell from lymph node of leukemic mouse. Cytoplasmic vacuoles are present containing several particles. Chrome osmium fixation. Monobasic lead acetate stain. × 52,000

FIGURE 9.—Shows single extracellular particle from lymph node of leukemic mouse. Details of internal structure are evident, including electron dense periphery of central nucleoid and relationship of two concentric peripheral membranes. Chrome osmium fixation. Modified lead hydroxide stain. × 157,000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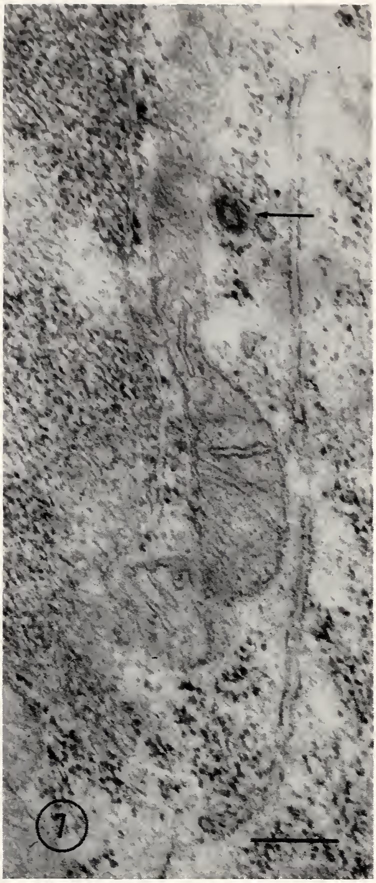
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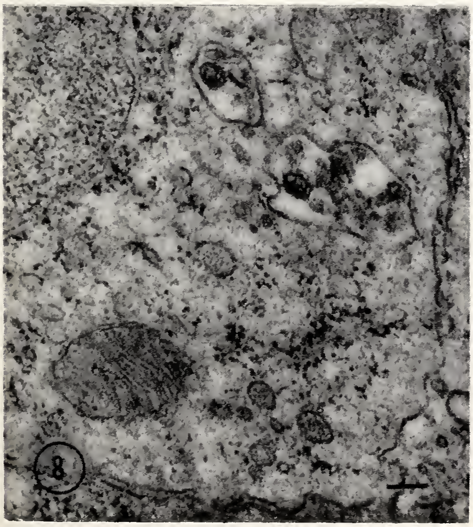
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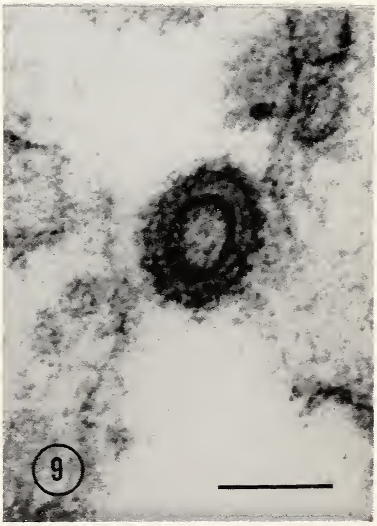
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Discussion

Dr. Gross (Veterans Administration Hospital, Bronx, New York): One of the main and very important questions is whether we have a single leukemia virus that is being recovered from mice in different laboratories by various means, or whether we are dealing with a number of viruses.

I think there are some differences between the viruses that we isolated and the one Dr. Moloney described, which will induce leukemia rather indiscriminately in different strains of mice, even in rats. Our attempts with Dr. Woolley to pass the leukemic filtrate into G.W. rats failed. This might have been caused by genetic resistance of that particular breed of rats used in our study.

The leukemia virus that we have been working with in our laboratory, which was originally isolated from AK spontaneous leukemia, is quite selective in its infectivity. It produces a high incidence of leukemia after inoculation into newborn mice of either the C3H/Bi strain, or into newborn mice of strain C57BR/cd. The leukemogenic potency of the agent was much less pronounced when mice of several other strains were used for bioassay, such as those of strains A, C57BL, BALB/c, etc. There is therefore among these two leukemogenic viruses a difference in their ability to induce leukemia in mice of different strains, not necessarily due to concentration of the virus. The leukemic filtrates used in our laboratory have a virus concentration resembling those used by Dr. Moloney.

There is apparently no difference in sensitivity to heat or in stability between these two viruses.

The leukemia virus with which we are working is widely spread in different organs of the injected animals. We found a considerable amount of virus in the blood plasma and a wide distribution in the thymus, spleen, kidneys, and leukemic tumors of mice with virus-induced leukemia. There is, however, only a small amount of the leukemia virus in the brains of leukemic donors.

I was under the impression that we were dealing with a pure lymphatic leukemia virus, since until recently we were able to induce consistently only lymphatic leukemia with this agent. More recently, however, we have observed that among newborn mice inoculated with passage A leukemia virus, and later thymectomized, many developed myeloid leukemia; a few developed chloroleukemia, and some erythroblastic leukemia. Filtrates prepared from such leukemias and inoculated into newborn C3H mice induced in most instances typical lymphatic leukemia. The results of these new experiments present a new problem.

Dr. Melnick (Baylor University): As an animal virologist I, as well as other virologists, almost invariably look for antigenic relationships between viruses even though they may have differing biological properties.

For example, among the ECHO viruses, the prototype Type 9 strain does not propagate in mice. However, a number of other strains have been isolated that do propagate in mice. With some isolates mouse pathogenicity is not a stable property. Upon isolation from nature they may not take in mice, but they can be manipulated in laboratories so they will gain this property. Antigenically, however, the mouse virulent and avirulent Type 9 strains are one and the same agent.

How much work relating to antigenic relationships has been done among these various viruses that produce leukemia? This may be a difficult question to answer, but there are means for its study other than to follow the development of the disease in these animals by performing elaborate cross-neutralization tests. For example, the simple complement fixation test, or other *in vitro* tests, might reveal relationships among these agents.

Dr. Moloney: Our agent is poorly antigenic. We have initiated some immunological studies in collaboration with Dr. Fink of our virus unit. We have been able to produce what we think may be an effective antiserum in rabbits.

Dr. Bryan: Perhaps Dr. Fink would tell us how the immune serums were produced in the experiments now in progress.

Dr. Fink (National Cancer Institute): The serums that are now under test were prepared in both rabbits and in BALB/c mice. The rabbits were immunized with Dr. Moloney's highly concentrated preparation of virus mixed with Freund's adjuvant. The animals received injections subcutaneously in two sites and were later given a booster injection. Similar preparations made from normal mouse spleens were injected into other rabbits.

The adult BALB/c mice were injected with either formalin-treated or heat-treated virus mixed with Freund's adjuvant. These animals received 2 injections at weekly intervals, either intraperitoneally or subcutaneously. They were then boosted with a very dilute viable virus and bled 3 weeks later. Other mice were inoculated with normal BALB/c spleen extract under similar conditions. Also included in the neutralization tests are normal serums from rabbits and BALB/c mice.

Dr. Bryan: Thank you, Dr. Fink. Dr. Gross, would you care to comment on what serological studies have been made of your virus?

Dr. Gross: The serum was inactivated at 56° C. for $\frac{1}{2}$ hour because we found that normal, uninactivated serum neutralized the virus promptly. We also observed, as Dr. Moloney did, that the leukemia virus is not very antigenic.

Dr. Bryan: Dr. Friend, would you care to tell us about your studies?

Dr. Friend (Sloan-Kettering): We have tested by neutralization tests, serums from the mice of Dr. Gross and serums from Swiss mice with leukemic mice of Dr. Schwartz. There is no cross-protection with our agent and these two. Antiserum against polyoma is also ineffective against our leukemia agent.

Unfortunately, the neutralization test is the only serological tool at our disposal. Our agent neither fixes complement nor does it hemagglutinate, and this becomes a very acute problem. But in spite of the fact that there does not appear to be any antigenic relationship at the moment between the seven new viruses, I think we might still be able to attempt a classification, probably by the use of physical and chemical properties that we now know.

Dr. Moloney has made a very nice attempt to correlate all the information available on the seven murine leukemias. It is striking that in morphology in the electron microscope most of these leukemia viruses are similar in size and in shape. It might be interesting to find a number of other characteristics that they have in common, for instance, sensitivity to ether—that is just one I might mention at the moment. I do not know if Dr. Moloney's agent is sensitive to ether.

Another possibility is that if the method that Dr. Moloney uses in concentrating the virus were used to treat other tumors, perhaps we might find or isolate other agents which might be related to some of the seven that we already have.

I would like to ask Dr. Moloney a question about the use of hyaluronidase in his technique. I noted that he not only uses it in the preparation of the tumor, but also in the dilutions for titration. I would like to know what the role of the enzyme is and about the titer of the agent—is it a high-titering agent or a relatively low one, as ours unfortunately is. And I would like to ask another question that has been raised many times before, and which has become more significant since the addition of this new leukemia virus: What is the relationship of the virus to the tumor from which it was isolated? In other words, how can we explain what a leukemia virus is doing in a sarcoma?

Dr. Bryan: Thank you, Dr. Friend. We are going to ask Dr. Moloney to answer these last questions after we hear from Dr. Schwartz. You mentioned that you had tested against antiserum with the Schwartz agent.

Dr. Friend: The antiserum against the Gross agent was rabbit antiserum and the antiserum against the Schwartz agent was not antiserum from rabbits but was from mice that had received the Schwartz tumor.

Dr. Bryan: Would you tell us your story, please, Dr. Schwartz?

Dr. Schwartz (Hektoen Institute): There are a number of points I would like to clarify. Dr. Moloney referred to our agent as being obtainable from neural tissue. This is quite true, and most of our work has been with brain filtrates, but we are able to get the agent from the tumor as well. Recently we reported with Dr. Sinkovics of our group that one of the reasons we are unable to obtain the agent from tumor tissue in the same concentration and with the same facility as from brain is the fact that there is an inhibiting factor present in tumor tissue extracts which can be separated rather easily from the active virus by a number of techniques.

There was some question about the oral route not being entirely oral because of injury to the esophagus during intubation. We have obviated that by allowing the animal to drink the brain extract or eat the whole brain without intubation, and, though the results are not quite as good as when the animal is intubated, they are quite striking, as Dr. Moloney has mentioned.

Perhaps the most interesting observation about the problem of the antigenicity of the agent is that even though the *in vitro* tests are not very striking, the neutralizing effect of the antibody is quite marked in *in vivo* experiments. For example, with our agent there is definite histologic evidence of leukemia 72 hours after the animals are inoculated with the virus. But if we start treating the animals at 72 hours with an antiserum prepared in rabbits, an antiserum that does not have a very high titer *in vitro*, the leukemic process may be reversed in a significant number of animals. If we allow these animals to go as long as a week after they have been inoculated with the virus, a time when they have gross evidence of leukemia, and treat them with the antiserum we are able to prolong their life about threefold. It would appear that the *in vivo* and *in vitro* effects are quite different as far as their presently measurable antigenic properties are concerned.

And finally, if I may anticipate Dr. Moloney's answer to Dr. Friend's question about hyaluronidase: In the passage of human leukemia to adult animals, we have used a technique not dissimilar to that used by Dr. Moloney, except that we were not using hyaluronidase in the preparation of our material. We found that if we used concentrated virus from the human brain we were able to induce leukemia in about 15 percent of adult animals of a resistant strain. But if we added hyaluronidase to this material, we were able almost to triple the incidence of leukemia and were able to get an approximately 50 percent incidence in a resistant strain of adult mice with material obtained from leukemic human brain.

Dr. Bryan: Are there any more comments on the specific phenomenon of neutralization?

Dr. Sabin (The Children's Hospital Research Foundation, Cincinnati, Ohio): I would like to ask Dr. Moloney whether we can assume that serums from mice and from rats that ultimately develop the tumors have not exhibited a neutralizing effect *in vitro*.

Also, is a normal serum an appropriate control for such a neutralization test? Is it possible—perhaps Dr. Moloney already has the data—that rabbits inoculated with normal mouse spleen, in which no agent is demonstrated, may have a similar effect if for no other reason than by aggregation of the particles in this particular extract. If so, would absorption of the antiserum that he has prepared eliminate this neutralizing activity?

I have only one other point to make: When there are such difficulties in demonstrating antibody, which may be present in very low concentration, it may be worthwhile to try a procedure that virologists used years ago with the varicella virus before tissue-culture techniques were available.

I particularly recall the work of Russell Amies who was able to show that when highly centrifuged concentrates of material containing the virus were mixed with serum of a patient it was possible to demonstrate specific agglutination of the particles.

Four years ago, at a meeting in the Soviet Union, I heard a report—and I regret that I do not recall the name of the man who made it—in which it was claimed that ultracentrifuged sediments of various tumors could be agglutinated by antiserum prepared against them and absorbed with normal tissue, and this was shown in the electron microscope. The particles were very small. He was attacked by others in the audience for his conclusion that this showed that these particles were virus particles.

They said it is possible they could be antigenic cell particles. I bring it up here as one of the techniques that would be interesting to explore, particularly with the serum of the tumor-bearing mice and rats described by Dr. Moloney.

Dr. Moloney: In answer to Dr. Friend's question about hyaluronidase, Dr. Schwartz relieved me of that. We use it for essentially the same reason.

On titers, we have a 100 percent leukemia incidence in the 10^{-4} dilution. I do not know what the 50 percent point is. What is the relationship of the virus to Sarcoma 37? We think of it simply as being a "passenger virus" in Sarcoma 37. Since 1908 this tumor has been in many laboratories. Apparently the Sarcoma 37 serves as an ideal medium for the maintenance of the leukemia virus, though not particularly for its multiplication. There is, however, multiplication to some extent. We have not been able to induce Sarcoma 37 with the leukemia virus, but only these lymphocytic neoplasms.

Graffi has expressed a similar opinion on the relationship of the myeloid leukemia virus to the many sarcomas and carcinomas from which he recovered it.

In answer to Dr. Sabin, our completed serological studies are still preliminary and not ready for publication; we have that work in progress. The reason why we did not publish the preliminary results was that normal serum is a very poor control, as Dr. Sabin has emphasized. We now have in experiment absorbed serum and serum produced against normal spleen extracts. The antiserum described here was produced against leukemic spleen extracts. We appreciate your last suggestion concerning our work.

No serological tests have been done on the rats.

Viral Activation of Cell Response [1, 2]

J. S. COLTER *and* **K. A. O. ELLEM**,[3] *The Wistar Institute of Anatomy and Biology, Philadelphia, Pennsylvania*

The title of this paper merits some examination. It was suggested to me, and it sounded so imposing that I could not bring myself to change it. Dr. Ellem and I have had ample time in which to regret that hasty decision. It seemed to us that the title suggested a discussion of possible mechanisms of viral tumorigenesis. We concluded that we were faced with two alternatives. We could have attempted to distill into a brief discussion the many excellent, but largely speculative, papers on this subject. We might then have given our paper the subtitle, "The Best of Dulbecco, Luria, Stanley, Latarjet, and Lwoff *et al.*"

The second alternative—and the one we have chosen—is something of a compromise. We shall permit ourselves the luxury of some speculation, but, in addition, present some of our observations concerning the interaction of a mammalian cell with a viral ribonucleic acid (RNA), and discuss some of the factors that influence the response of the cell to the nucleic acid. The ribonucleic acid in question is not of tumor-virus origin, a fact which may seem to reflect a departure from the spirit of this symposium. However, we do not feel that this is true. Oncogenic viruses may in some cell types and under certain conditions act as cytocidal agents. Continuous replication of a virus without destruction of the host cell is a feature apparently not confined to tumor viruses. For the moment we would suggest that the basic mechanisms in the response of a cell to a tumor virus may be substantially the same as those involved in the response to a non-tumor virus. Thus a non-tumor virus system might be considered as a model that could provide a baseline for investigations of the special characteristics of tumor-virus systems.

The now classical experiments of Hershey and Chase (*1*) provided the first demonstration of the unique importance of bacteriophage deoxyribonucleic acid (DNA) in the reproduction and genetic continuity of these viruses. The corresponding central role played by the RNA of the tobacco mosaic virus (TMV) was conclusively and directly demon-

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[1] Presented at a Symposium on Phenomena of the Tumor Viruses, New York, N.Y., March 25 and 26, 1960.

[2] A portion of the work presented was supported by Public Health Service research grant C-4534, Public Health Service training grant 2G-142, and the Samuel S. Fels Fund.

[3] Traveling Fellow of the New South Wales State Cancer Council.

strated by Gierer and Schramm (*2*). That the genetic information contained in the TMV particle for the determination of the progeny virus lay in the nucleic acid core, and not in the protein coat, was also deduced by Fraenkel-Conrat and coworkers (*3, 4*). By mixing the nucleic acid of one strain of TMV with a preparation of protein from another strain, they effected reconstitution of viral activity. Progeny arising from an active infection initiated by this artificial virus always exhibited the characteristics of the virus from which the nucleic acid had been derived.

The results of Gierer and Schramm were quickly extended to a number of animal viruses. Colter *et al.* (*5*) demonstrated the infectivity of RNA solutions isolated from tissues infected with poliomyelitis, West Nile, and Mengo encephalomyelitis viruses. Alexander *et al.* (*6*) reported the successful isolation of infectious RNA from type I poliomyelitis virus. Since then, the list of viruses from which biologically active RNA has been isolated has grown rapidly. I shall not undertake to reproduce it here, nor will I recount the various criteria by which RNA has been distinguished from the corresponding virus particles. It may be worth noting, however, that the viruses which make up the list are all small and are viruses with either neurotropic or myotropic potentialities.

A number of laboratories are involved in studies designed to demonstrate the biological activity of nucleic acids from tumor viruses. In addition, there is renewed interest in attempts to isolate viral agents from human tumors. As a part of this latter program, considerable attention is being given to nucleic acids isolated from tumor specimens—an attention which has no doubt received considerable impetus from the observations of Holland *et al.* (*7*) that viral RNA has in some cases a wider spectrum of activity than has the native virus. It would seem then to be of some value to consider certain factors which influence the interaction of RNA with the host cell.

In our laboratory, an examination of the optimal conditions for the interaction of Mengo RNA with strain L mouse fibroblast cells has been made (*8*). In brief, we have used the following system: Strain L cells are incubated, as a suspension of single cells, with Mengo RNA. After incubation, the cell-RNA mixture is diluted tenfold with normal growth medium and applied to preformed monolayers of strain L cells. After 1 hour the medium is removed and the monolayer, with the infected cells affixed thereto, is overlaid with agar. Plaques are read after 2 and 3 days.

It was found that the number of infectious centers formed in mixtures of RNA and strain L cells is a function of the osmotic pressure of the medium in which cells and nucleic acid interact. Maximum efficiency of infection was obtained in solutions of sodium chloride or in solutions of sucrose in physiological saline, when the osmotic pressure of the diluent was 4 times that of physiological saline. The number of infectious centers formed in the optimal sodium chloride or sucrose solutions was 20 to 30 times the number formed in physiological saline.

The infectivity of intact Mengo virus in a similar suspended cell system was adversely affected by increasing the osmotic pressure of the extracellular medium. The number of infectious centers formed from virus was reduced to less than 0.2 percent by an increase in the concentration of sodium chloride to 0.4 M and to 3 percent by the addition of sucrose to 0.7 M.

Studies are in progress which we hope will elucidate the mechanism whereby solutions of high osmolarity stimulate RNA infectivity. The data suggest that such solutions act not by affecting the RNA itself, or by stimulating the uptake of the nucleic acid, but rather by creating a hypertonic intracellular environment more suitable to the stabilization of the absorbed infectious RNA unit. It has been suggested that the increased RNA infectivity in solutions of high ionic strength may be due to the inhibition of intracellular ribonucleases (*9*); ribonuclease is known to be inhibited by solutions of high ionic strength (*10*). Another possible mechanism is suggested by our preliminary observations that the infectivity of Mengo RNA is reduced by ribonuclease-free proteins when nucleic acid and protein are mixed in solutions of physiological ionic strength. At higher ionic strengths inhibition does not occur. A hypertonic intracellular environment could then act by preventing nonspecific immobilization of the viral RNA by cytoplasmic proteins.

The period for which RNA and cells are incubated is quite critical. In the Mengo RNA-L cell system, at 37° C., and in the optimal sodium chloride or sucrose media, maximum production of infectious centers is obtained with a 15-minute incubation period. The number of infectious centers formed drops precipitously when incubation is continued for longer than 15 minutes. This probably reflects a loss of viability of the strain L cells in solutions of high osmotic pressure, and is very likely a function of the particular cell being used.

Infectious RNA is perhaps most readily tested in an *in vitro* system. Extracellular ribonuclease can be removed by washing; the conditions under which cells and RNA interact can be carefully controlled, and the results can readily be quantitated. *In vivo* demonstration of activity is not likely to be successful unless the target organ is readily accessible so that the nucleic acid has a chance of immediate contact and entry into the appropriate cells types. Infectivity of viral RNA has been demonstrated in the mouse only when the nucleic acid is injected directly into the brain or muscle. RNA from the virus of foot and mouth disease, for example, is inactive when given by intraperitoneal injection, but will produce an infection when given by the intramuscular or intracerebral route (*11*). We have found that the Ehrlich ascites tumor cannot be infected by the intraperitoneal injection of Mengo RNA into tumor-bearing mice. However, these cells can readily be infected by removing them from the animal, washing them several times in a balanced salt solution, and exposing them to the RNA in the absence of serum. It may be of significance with regard to these observations that, among mouse tissues, muscle, brain, and cells of the Ehrlich ascites tumor are exceptional in their low level of intracellular ribonuclease (*12*). It would be of interest to know the levels of intracellular ribonuclease in the various primary explanted tissue cultures used in this work. Preliminary estimations in our laboratory on some stable cell lines suggest that they, like all ascites tumors that we have examined, have very low levels of ribonuclease activity at physiological hydrogen ion concentrations.

A potentially active viral RNA must then overcome many obstacles before it can initiate an infection in an experimental animal. Degradation by extracellular—and perhaps intracellular—ribonuclease is an ever-present hazard, as is nonspecific immobilization by proteins. To this, one must add the inherent thermal instability of the RNA molecule. At 37° C., and in the presence of 0.001 M Versene, Mengo RNA has a half life of more than 90 minutes. In the absence of Versene, it has a half life of not more than 30 minutes.

These considerations concerning the *in vivo* lability of viral RNA may also apply to DNA. Although unpurified transforming principle has been found to be stable for at least 24 hours in the mouse (*13*), deproteinized preparations, while still effective *in vitro*, will not transform *in vivo*.

Of the several reports concerning the infectivity of DNA extracted from virus-infected tissues, the most convincing is the recent one by DiMayorca *et al.* (*14*). These investigators used the duponal method (*15*) and the phenol method of Kirby (*16*) to deproteinize concentrates of tissue cultures infected with polyoma virus. The preparations produced cytopathogenic changes in primary cultures of mouse-embryo fibroblasts accompanied by the appearance of deoxyribonuclease-insensitive polyoma virus, identified by its ability to produce tumors in the hamster. The activity of the nucleic acid preparations was abolished by deoxyribonuclease, but was unaffected by ribonuclease.

While no other tumor virus has yielded an infectious nucleic acid, there seems no reason to believe that other viruses which have been studied should prove any exception to the basic notion that the viral genome is contained within the structure of the nucleic acid. Tumor viruses cannot be grouped together as DNA-containing particles. While the polyoma, Shope papilloma, and rabbit fibroma and myxomatosis viruses apparently belong to this group, the best preparations of the Rous sarcoma (*17*) and myeloblastosis (*18*) viruses have been found by chemical analysis to contain only RNA.

There appear to be many aspects in common between the biology of tumor and non-tumor viruses. The differences between them are mainly quantitative rather than qualitative (*19*). The major distinguishing feature between them—the ability to produce tumors in animals—reflects only one aspect of the virus-cell relationship. Thus the polyoma virus, which produces multiple tumors in rodents, kills mouse-embryo fibroblast cells *in vitro* with the release of large quantities of virus from the dying cells. This agent also induces *in vitro* a proliferation of fusiform cells in hamster-embryo cultures (*20*) during which production of the virus is poor. A similar relationship between cell proliferation and virus production is seen in the Shope papilloma virus. Proliferation of the basal layer of the rabbit epidermis is accompanied by low virus production, whereas increased virus production occurs in the nonproliferating, keratinizing layer (*21*).

In an analogy with the behavior of virulent as opposed to temperate phages, Dulbecco (*19*) has called the "cytocidal-high virus yield" relationship between virus and cell nonintegrative, and the "cell proliferative-low virus yield" relationship integrative. He points out that the important deviations from the temperate phage-bacterium relationship are: (1) Virus-induced tumors usually contain either virus-specific protein or intact virus particles, and (2) instead of the discontinuity which exists between the prophage and the vegetative state in lysogenic cells, there is an apparent continuity of the changes in the properties of virus-carrying cells.

The observation that different strains of Rous sarcoma virus can produce consistent, hereditable changes in the morphological appearance of chicken fibroblasts is important (*22*). In this case an RNA virus has converted its host cell with respect to at least three hereditable characteristics: (1) the ability to produce Rous sarcoma virus of the type that caused the infection, (2) the property of stability to subculture, and (3) a change in the morphological appearance of the cell—a change not determined exclusively by the 2 preceding characteristics, since cells possessing these by virtue of infection with different strains of the Rous virus may have different and equally unique appearances. This phenomenon of conversion is not the prerogative of tumor viruses.

Infection with cytocidal viruses may also result in conversion. As examples, one may cite the acquisition of hemagglutinin on the surface of influenza-infected cells (*23*), the production of interferon accompanying influenza-virus multiplication (*24*), the protein factor produced during adenovirus replication which induces cytopathic changes in HeLa cells (*25*, *26*), and the virus-induced formation of a number of enzymes related to the special requirements of T2 phage DNA synthesis in *Escherichia coli* (*27*, *28*).

Although conversion by non-tumor viruses is not heritable when the agents are cytocidal, in the sigma virus of *Drosophila*, which is responsible for CO_2 sensitivity in this fly, the evidence for a hereditable phenotypic change in the organism seems indisputable (*29*). The evidence so far obtained with the Rous sarcoma virus suggests that the ability to produce virus is transmitted to the progeny of an infected cell as an intracellular event, the virus continuing to be produced at a slow rate in succeeding generations (*30*). The moderate behavior of these viruses suggests a stable addition to the genetic information of the infected cell, in line with Luria's concept of viruses as infective genetic materials (*31*). In a sense then there has been an addition to the genotype of the cell.

The apparently uncontrolled production of viral components may be viewed in terms of the genetic control of the host cell. The replication and expression of a normally occurring nucleic acid is undoubtedly the result of a fine balance betweeen activating and suppressor controls. The nucleic acid of a virus carries new information which is sufficiently intelligible to the cell to misinform it, but which is sufficiently foreign so that the cell has no control or suppressor systems for it. Consequently the viral nucleic acid and other viral constituents are synthesized at the cost of disturbing the homeostasis of the cell. Integration of a virus may mean greater recognition of the viral information, and this may be reflected in partial control and a degree of compatibility between the viral and host genomes. Nonintegrative relationships perhaps imply little or no recognition and consequently uncontrolled and deleterious viral multiplication.

The genetic information of the virus may be stored in the converted cell in at least two ways: (1) The viral nucleic acid may persist as such, replicating at least as fast as the cell, and exerting an influence on the cell phenotype more or less directly via the abnormal proteins it determines.

For the RNA viruses, models exist in some of the cytoplasmic factors in sexual and cellular heredity (*32*) and perhaps in the microsomes of normal cells, while, for DNA viruses, a parallel is seen in the Kappa particles of the paramecium. However, the limited amount of genetic information which, on current hypotheses, it seems reasonable to expect in the small and medium-sized viruses (*33*) may incline us to view a detached seat of power, relatively independent of the host genetic apparatus, with some intuitive feelings of misgiving.

(2) A fashionable alternative is to consider the possibility of integration of the viral nucleic acid with the host genome. In this regard it is of interest to recall the radiological data of Rubin and Temin (*34*). They found that the ability of chick fibroblasts to produce Rous sarcoma virus and their ability to divide were equally sensitive to X-ray inactivation. The capacity for another virus of a cytocidal nature in this cell was many times more resistant to irradiation. They felt that this evidence suggested that a necessary early step in Rous sarcoma virus replication involves integration with the host genome, and that a common radiosensitive target controls the ability of the cells to divide and their ability to produce Rous sarcoma virus.

As a means of incorporating information more or less permanently into the host cell from viruses containing DNA, the model of lysogeny exists.

In the RNA viruses it is conceivable that transfer of genetic information could take place from viral RNA to host DNA by a mechanism akin to that postulated by Stent (*35*) for the replication of phage DNA. On the basis of P^{32} suicide and utraviolet inactivation data, and of a copy choice mechanism for DNA synthesis, he postulated that a ribonucleoprotein (RNP) strand was synthesized in the deep groove of the parental DNA helix, a ribonucleoprotein which carried the imprint of the DNA duplex, since the base pairs of the latter would select its constituent ribotides. The newly synthesized RNP molecule would then unwind itself from the parent DNA duplex and remain independent until meeting and "mating" with an identical macromolecule with which it would entwine to form an identical duplex. This structure could then serve as a template for the synthesis of DNA, a complementary pair of deoxyribotides being attracted by the single RNA base.

The studies of Doudney and Haas (*36*) on the production of stable bacterial mutants by ultraviolet irradiation have provided data which, by suggesting an important role for RNA in the replication of DNA, are at least compatible with Stent's hypothesis. These investigators demonstrated a close correlation between postirradiation RNA synthesis and mutation fixation. The latter was interrupted if RNA synthesis was disturbed, and was complete before any DNA synthesis occurred. They suggested that abnormal pyrimidines were formed in the metabolic pools by the ultraviolet irradiation, and that these were incorporated into ribonucleoprotein, which in turn imprinted faulty information in the base sequence of DNA synthesized on it.

Stent's postulates could explain how genetic information might be transmitted from DNA to RNA and thence back to DNA. For a similar mechanism to operate for an RNA virus, at least three conditions would have to be met: (1) The active viral nucleoprotein or RNA would have to be in proximity to the nuclear apparatus. All available evidence suggests that the nucleoprotein of the small and medium-sized RNA viruses is synthesized in the nucleus. (2) At least two viral genomes would have to be present in the cell to form an identical RNP duplex. Since one virus particle is sufficient to initiate an infection (*30*), some viral replication would have to proceed before this RNP duplex could be formed. It follows from this that DNA synthesis cannot be a prerequisite to viral replication, but merely an incidental phenomenon. (3) There would have to be considerable homology between the base ratios of the hypothetical DNA and the host DNA which the viral nucleic acid would determine. It may be pertinent that, in bacterial systems, successful genetic recombination has been observed only between organisms containing DNA's of the same base ratio group, and the same sort of "code similarity" is probably required for the successful integration of a phage or prophage with a bacterial chromosome (*33*).

A corollary arising from this hypothesis is that it might be possible to isolate from some virus-induced tumors both DNA and RNA capable of transmitting the biological properties of the intact virus.

The new nucleic acid added to the host genome might influence other genes in a fashion similar to that seen in the alteration in the activity of loci near the site of attachment of certain genetic elements to the chromosome, such as the fertility factor in *E. coli* (*37*) or the controlling elements in maize (*38*). It may also perhaps induce host modifications as an intrinsic property of its own genetic constitution. As examples, one may cite the toxin production in *Corynebacterium diphtheriae*, which is inextricably related to a phage infection (*39, 40*) or in the suppression by λ prophage of the multiplication of certain mutants of the T-even phages (*41*). This would in effect be somatic mutation, as has been pointed out by Luria. Successive somatic mutations have been suggested as the basis for the evolution of the cancer cell (*42, 43*) and may be the final common pathway for the activity of many carcinogens.

The foregoing considerations suggest a mechanism by which a hereditable defect might be built into a mammalian cell—the introduction, by a viral agent, of an abnormal ribonucleoprotein, or the intracellular synthesis of such an abnormal molecule after irradiation. The consequent alteration in DNA could lead to deletions, resulting, for example, in a loss of systems responsible for negative feedback on the specific enzyme-forming systems required for cell division (*44*), or the gain of abnormal potentialities in differentiation (*45*) and other phenotypic expressions of malignancy.

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Discussion

Dr. Stanley (University of California): Dr. Colter has made a beautiful summary of the implications of infectious nucleic acid, and I can add very little. I would like to call the attention of this group to some of the new work that is coming out in the plant virus field, starting with Dr. Gierer and Dr. Mundry's work in Tubingen, Germany, on artificially produced mutants with the use of nitrous acid, in which they showed that through the conversion of cytosine to uracil by nitrous acid treatment they obtained mutants. Other workers in Tubingen found no changes in the protein, which makes you wonder a little bit about the message that is carried in the nucleic acid.

We have made similar investigations in our laboratory and we do find changes. You may remember Dr. Knight's original observation that the last amino acid on the tobacco mosaic virus (TMV) polypeptide subunit is threonine which can be removed very easily with carboxypeptidase. All the TMV strains so far examined have this characteristic.

One of the mutants which has been obtained by nitrous acid treatment does not have this characteristic, and we already know that the changes in the amino acid sequence of a TMV polypeptide is presumably brought about by the conversion of one base into another base—cytosine into uracil. We think this is the first of the unraveling of the code that is contained in the sort of thing Dr. Colter has been talking about.

You need only another hundred or so such relationships between the polynucleotide chain and the polypeptide chain to know why a particular nucleic acid can be self-replicating. I think this will come with time, and that this work on viral nucleic acids is extremely important.

Of course from the standpoint of this group the observation, which I believe was initiated in Dr. Syverton's laboratory, that the nucleic acid will grow to a certain extent in cells which are not susceptible to the intact nucleoprotein is something to be kept in mind.

The implications of the work of Shope and his colleagues with the papilloma virus, as well as certain other work, tend to indicate that the viral nucleic acid probably can move as free nucleic acid. It is quite terrifying to think about, because everybody who is interested in immunology is sort of left behind the eightball, so to speak, until somebody introduces a new technique that will tie something onto nucleic acid and will enable formal immunology to be effective with this particular probable infectious process.

I think the business of integrative and nonintegrative viruses is something that should be kept in mind—this is a good concept, and I was glad to hear Dr. Colter again remind you of it.

Dr. Barricelli (Vanderbilt University): I would like to stress the point that the incorporation of a virus in the genetic material of a cell may indicate the existence of a crossing mechanism by transduction in cancer cells.

According to Dr. R. S. Fisher's results, a crossing mechanism would make it possible to increase the rapidity of evolutionary adaptation of a species by a large factor—a factor roughly comparable to the number of genes involved. It is obvious that the rapidity of evolutionary adaptation, resulting from a crossing mechanism, is essential to explain the rapid formation of a population of cells different from other cells of the body and adapted to overcome the defenses of the organism. The rapid increase in resistance of tumor cells to drugs and other therapy would also be easier to interpret by a crossing mechanism.

The question arises, therefore, whether the primary difference between normal and cancer cells may consist in the acquisition or uncontrolled increase of a primitive ("atavistic") crossing mechanism. Depending on the type of cancer, crossing may occur by any one, or by several, of the three mechanisms which are known in bacteria: (1) transformation; (2) transduction; (3) so-called "regular crossing of K_{12} bacteria." According to this theory, only cancers which cross by transduction can be transmitted by viruses from cell to cell. Moreover, a latent virus recently evoked by a mutation into a virulent stage could hardly be expected to have developed or restored the ability to survive outside the body and would hardly be infectious. This would explain the lack of infectivity which seems to be a common characteristic of most tumor viruses.

Preliminary work (selection of genetic markers) to investigate the possibility of performing crossings with human synovial cells is being carried on at the New York University-Bellevue Medical Center by Dr. L. N. Chessin under the leadership of Dr. K. Hirschhorn.

Dr. McLaren (University of Minnesota): I would like to compliment Dr. Colter and Dr. Ellem on their excellent paper.

I would like also to comment briefly on Dr. Colter's reference to our work which indicated that RNA from enteroviruses had a wider host cell range than intact virus. Whether our observation has any immediate application to the isolation of viral agents from human tumors is yet to be learned. Infection of various naturally insusceptible cells, such as rabbit cells, with enterovirus RNA *did* result in production of virus; however, this progeny virus was not distinguishable from the virus from which the RNA was extracted. This strengthened our previous conclusion that enterovirus specificity was dependent upon interaction between viral protein and receptors on susceptible cells. If the receptor mechanism was bypassed by the use of viral RNA, we could infect insusceptible cells. No cytopathic changes were observed, probably because so few insusceptible cells were infected. We had to rely on a susceptible assay system to detect virus multiplication. If these findings have application in studies of the phenomena of tumor viruses, we must have sensitive assay systems for intact tumor viruses and tumor virus nucleic acids.

I have two questions I would like to ask Dr. Colter:

(1) With reference to your statement concerning low levels of intracellular ribonuclease in mouse brain, muscle, and Ehrlich ascites tumor cells, what cells have been found to contain high levels of ribonuclease and are these cells more resistant to infection by viral RNA *in vitro*?

(2) You mentioned that infectious RNA has been prepared from only small animal viruses of either neurotropic or myotropic potentialities. This is an interesting correlation which was not obvious to me. Maassab reported last year that infectious RNA was obtained from influenza virus-infected cells. We have tried without success to demonstrate infectious nucleic acid from high titer influenza viruses, and I was wondering what has been the experience in your laboratory with infectivity of nucleic acid from influenza or other myxoviruses.

Dr. Colter: In regard to your question about the intracellular ribonucleases, we have not made a frank attempt to correlate the vulnerability of a cell to infection with RNA with its level of intracellular ribonuclease. A scanning of the literature, however, led me to suggest that there may be this relationship. Certainly it is true that in the mouse only brain and muscle have been successfully infected with naked RNA. There are all kinds of tissues in the mouse that have active intracellular ribonucleases. Nearly all tissues, with the exception of muscle and brain—liver, kidney, salivary gland, lymph nodes, spleen, etc.—have relatively high levels of intracellular ribonuclease.

We have made no attempt to infect any of these tissues in culture *in vitro*. I am not sure that anyone else has either.

In answer to your question about the myxoviruses, we have not attempted to isolate RNA from the myxoviruses. In assembling this dissertation, I did not consider the report by Maassab, for one reason: It seems to me that to talk about infectious RNA, the number one job is to recover the virus and show that it is identical with the virus from which the RNA was obtained; this Maassab did not do.

Dr. Miroff (University of North Dakota): We have infected HeLa and ascites cells with poliovirus and followed the metabolic alterations with radioisotopes. This cytolytic virus produces a marked increase of nucleic acid and phospholipide synthesis. These alterations have been shown with the use of cytochemical techniques.

To infect ascites cells, which have been grown continuously in agent-free mice with the mammary-tumor agent, produces no detectable effect upon the nucleic acids, but it does alter phospholipide metabolism. This would lend support to the idea that the agent enters the cell, integrates with it, and produces a metabolic alteration, but unlike cytolytic virus does not alter nucleic acid metabolism.

Dr. Rubin (University of California): I should like to say a word about the morphologic alteration of chick cells to sarcoma cells after infection *in vitro* with Rous sarcoma virus. When we expose cells to a high concentration of virus, we observe characteristic morphologic alterations in some cells in 2 days, and in practically all cells in 3 to 4 days. In studying the multiplication of the virus under the same conditions, we find some cells initiating the production of mature virus in 2 days, but most cells initiating virus production at 3 or 4 days. Therefore, a close association appears to exist between the onset of virus production and morphologic change in the cell. This would suggest that if any analogy with temperate bacteriophages is valid—and it may be that none is—it is more likely to be an analogy with lysogenic conversion which is the consequence of virus multiplication, rather than transduction which results from the integration of new genetic material.

Radiation studies on Rous sarcoma virus infection have been amplified recently. As reported previously, cells irradiated with small doses of X ray lose their capacity to produce Rous sarcoma virus if exposed to the virus after irradiation. This is in sharp contrast to the radioresistance of the capacity of the same cells for the production of cytocidal viruses.

The high degree of radiosensitivity of cellular capacity for Rous sarcoma virus production is obtained only when the cell is infected with a single infectious unit of the virus. If the cell is infected with several infectious units, the capacity is relatively radioresistant. The significance of this multiplicity effect is not yet clear, but a similar observation has been recorded in work on the capacity of bacteria to support growth of the temperate bacteriophage lambda.

Even in single infection with Rous sarcoma virus, the ability of the cell to produce virus becomes radioresistant about 20 hours after infection. Therefore, if we assume that the early radiosensitivity is in fact a reflection of the need for the viral and host functions to become integrated (in the broad sense of the word) we must assume that the integration is completed within 20 hours.

Dr. Beard: Dr. Colter, I understand that it is your idea that the induction of the state of malignancy in these cells is associated with the change in genetic constitution, so that fundamentally the major alteration in the cells, which contributes to their malignant attributes, is this genetic change.

Dr. Colter: I think that is a fair statement of my position. What I have attempted to do is to indicate some of the ways in which that change can be induced, starting with DNA and RNA viruses.

Dr. Beard: There is a great possibility that this cannot be considered as a generalization with respect to the virus-induced tumors. It should be considered, after Dr. Bonar gives his paper, that we have seen no evidence which might indicate a genetic change in the cells of avian myeloblastosis. From this point of view, we feel quite definitely, one should be able to conceive that this process of so-called malignancy, or the exhibition of the attributes of malignancy, does not necessarily have anything to do with the change in the genetic constitution of the cell, or, to put it another way, it is not necessarily associated with mutation of the cell. In this system the myeloblasts would go on multiplying forever if one wished to maintain the cultures. Virus is transmitted from one daughter cell to the other as cell growth continues, and after 2 years the cells still look as they did in the beginning. As far as we can see without doing intimate chromosomal studies, they retain the same characteristics.

It would appear that the apparent neoplastic attributes of the myeloblasts might well be regarded as no more than an interference with the normal processes of maturation of the cells by means of which they are converted ultimately to white blood cells of the blood circulation and tissues.

Dr. Colter: I felt quite sure you were going to raise this question of myeloblastosis. I would say only that you do have a rather unique cell, and, though there is no morphologic evidence of a transformation, the myeloblasts clearly have been transformed with respect to two characteristics: (1) the capacity to support virus replication, and (2) the capacity to grow continuously in tissue culture. These are not characteristics of the normal myeloblast, so there has been, in effect, a transformation of some sort.

Dr. Black (University of Illinois): In connection with this discussion, I think the work of Dr. Armin C. Braun, of the Rockefeller Institute, on the non-self-limiting, autonomous plant tumor, crown gall, is particularly pertinent, and I would like to describe briefly his experiment. A plant tumor, crown gall, grown in culture, is obtained in a state where the cells are separated from one another. Individual cells are isolated, allowed to grow into groups of a certain size, and then grafted onto tobacco plants. In the early stages, under certain conditions, the grafted tissue shows tumor characteristics of a teratoma. If the grafts are treated in a certain way, the tissue reverts to form a normal top which will flower and produce normal seed. Thus, starting from a single crown gall cell (a plant cell that normally remains tumorous) a completely normal plant can be regenerated by a specific treatment. It seems to me that this experiment demonstrates that crown gall is not the result of a somatic mutation. (*See* Proc. Nat. Acad. Sc. 45: 932–938, 1959.)

Constitution of Tumor Viruses[1]

M. A. EPSTEIN, ***Bland-Sutton Institute of Pathology, Middlesex Hospital, London, W.1, England***

Besides being of fundamental importance, the question of tumor virus constitution raises a number of related issues each of which is highly interesting in its own right. These fall into two main groups, one, perhaps the more pedestrian, being concerned with problems of methodology and technique, and the other with basic biological concepts and the exciting speculations to which, in our present state of knowledge, their consideration leads.

The practical problems involved are not peculiar to the study of tumor viruses but are those which apply throughout the virus field and, in that context, they are both familiar and obvious. Nevertheless it is worth recalling them at the present time for two reasons: (1) Recent discoveries (*1–5*) have led to an enormous expansion in the study of tumor viruses and to the recruitment of many new workers, and (2) we are clearly at the start of an era in which the search for tumor agents will be extended to human neoplasia (*6*, *7*). As this extension goes forward, experimental work cannot be too carefully controlled or too cautious, for one will be dealing with an emotionally explosive and sensational subject.

The actual nature of the practical considerations at issue is, however, straightforward; there is the problem of virus identification, the problem of virus isolation, and, finally, the problem of determining virus composition.

The present communication is concerned mainly with virus composition, but, before one can investigate this in a given virus, the agent itself must be identified. The recognition of so-called "virus-like" particles with the electron microscope is no more than a preliminary step, for this instrument alone can never serve to identify a virus, distinguish it from another morphologically similar virus, or establish its activities. When particles are found in association with a particular neoplastic condition, they must be linked to some sort of biological activity before any conclusions can be drawn as to their nature. Evidence is constantly accumulating which indicates that a wide variety of viruses can be and are present in normal cells (*8–10*), thus suggesting that certain tissues support a normal viral flora analagous to the bacterial flora of healthy individuals. This being so it is more than ever necessary that if virus-like particles are detected in tumors, they should be shown to be there in an active capacity and responsible for the disease process, rather than as casual loafers merely looking on. To this end, morphological investigations must be integrated with biological tests; if this is done the contribution of morphology in this field can be vital, whereas, alone, its usefulness is restricted and may even be seriously misleading.

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[1] Presented at a Symposium on Phenomena of the Tumor Viruses, New York, N.Y., March 25 and 26, 1960.

The second practical question requiring consideration is that of virus isolation. Exact information on the composition of a virus can be reliably obtained by chemical analysis, but for this complete purification is a prerequisite which in itself can be troublesome to assess and extremely difficult or even impossible to attain. On the other hand, information on virus composition can also be obtained, and this where only partially purified material is available, if cytochemical digestion methods are used and their effects on individual virus particles observed with the electron microscope. But whichever of these approaches is used, the necessity for isolating a given virus remains, so that the requirement regarding purification for either technique is merely one of degree. In addition, experiments of quite a different kind can also throw light on viral composition. Where protein-free extracts of viral nucleic acid can be shown to possess infectivity, the type of nucleic acid involved may be demonstrated by abolishing the infectivity with a known specific nuclease.

Turning to the actual determination of virus constitution, relatively little has been achieved so far with true tumor agents. Recently, however, the virus of avian myeloblastosis has been found to occur in enormous quantities in the blood of infected chickens and has been successfully isolated from this source by differential centrifugation (*11*). Bonar and Beard (*12*) have applied microchemical methods of analysis to material obtained in this way and have shown that the virus contains nucleic acid of the ribose type and that in other respects its chemical constitution is comparable to that of the generality of infectious viruses.

For our own work with the Rous virus we have preferred to rely on thin-sectioning techniques for electron microscopy, coupled with specific enzymic digestions. As has already been pointed out, this approach can be used without complete purification of the virus and in addition it has a further advantage. Chemical analysis can show of what a virus particle consists as a whole, but it cannot give information about the composition of morphological structures in the particle. This type of knowledge can only be obtained by examining these structures *in situ*, and combined electron microscopy and digestion methods provide a way of doing so.

The starting material for the experiments consisted of suspensions of Rous virus prepared from egg-grown nodules of Rous tumors by treatment with a fluorocarbon; the treatment freed the suspensions of almost all formed host substances, but certain other host-cell contaminants remained (*13*). However, when the suspensions were subjected to high speed centrifugation, pellets resulted in which characteristic areas were composed almost entirely of uniform particles with which tumor-producing activity could be shown to be associated (*13*).

Virus-containing fragments of such Rous pellets were fixed with potassium permanganate (*14*) and prepared directly for thin-sectioning for electron microscopy. When examined in this way the virus was found to be a spherical particle about 75 mμ in diameter, with a central dense nucleoid surrounded by a less dense outer zone or viroplasm, the whole being limited externally by a double membrane (fig. 1).

Besides being an excellent fixative for the electron microscopy of viruses, potassium permanganate has the further important attribute, unlike the more commonly used osmium fixative (*15*), of allowing subsequent digestion of nucleic acids by appropriate specific nucleases (*16*). In further experiments virus-containing samples were treated with ribonuclease; they were first fixed, as before, with potassium permanganate and then incubated for 2 hours at 37° C. in 30 percent alcohol containing 0.1 percent by weight of thrice-crystallized, protease-free ribonuclease. At the same time control samples were incubated in alcohol without enzyme. All samples were then dehydrated, embedded, and sectioned for electron microscopy. The enzyme digestions were applied in an alcoholic medium, in order to adhere closely to the series of treatments recommended by Luft (*14*) for the best possible morphological preservation of specimens. It was known, of course, from preliminary experiments (*16*) that both types of nuclease were fully active under such conditions.

Examination showed that the incubation procedure made the particles appear ragged and extracted as compared to samples fixed and prepared directly (fig. 1). Nevertheless, in the control preparations, which were not exposed to ribonuclease (fig. 2), the nucleoids and outer-limiting membranes of the particles remained intact. In marked contrast, the nucleoids were digested from the particles which had been subjected to the ribonuclease (fig. 3), and these particles were also slightly swollen as if some central bracing structure had been removed. Thus, it can be concluded that the Rous virus contains a substantial amount of ribonucleic acid and the methods used have demonstrated that the site of localization of this material is the nucleoid.

Although the specificity of the ribonuclease and the findings of the control experiments permit these results to stand on their own, it was thought advisable to attack the question from an additional and unrelated angle. While the electron microscope preparations were being made, other samples of the virus-containing regions of Rous pellets were smeared on glass slides and allowed to dry. The smears were then fixed and stained with acridine-orange for nucleic acids, the staining being done either directly, or after incubation for 2 hours at 37° C. in ribonuclease, or after similar incubation in control medium without the enzyme.

When structures containing ribonucleic acid stained with acridine-orange are examined in ultraviolet light, they usually exhibit an orange-red fluorescence, which specifically identifies the presence of ribonucleic acid if it is abolished by ribonuclease digestion before the staining (*17*, *18*). Although light microscopy obviously cannot resolve individual Rous virus particles, electron micrographs of sectioned pellets had established that the material used in the acridine-orange staining experiments consisted almost wholly of the virus (fig. 4). Thus, the over-all fluorescence response obtained with and without ribonuclease digestion can be taken as a reflection of the response of the individual particles to the acridine-orange staining.

The presence of much ribonucleic acid in the Rous virus was therefore confirmed by the intense orange-red fluorescence of the virus when stained directly, by the absence of such fluorescence when stained after ribonuclease digestion, and by the retention of the orange-red fluorescence in the control preparations which had been treated with enzyme-free medium alone (*16*).

To digress briefly, it is interesting to note that this type of integration of electron microscopy with cytochemical methods is not something which can be applied only to the Rous virus and its ribonucleic acid. It has wider applications, both in the tumor virus field and in the field of virology in general, as has been shown by a recent similar study of an infectious virus containing nucleic acid of the deoxyribose type (*19*).

The experiments just described give good evidence that another tumor virus, in addition to the virus of avian myeloblastosis, namely, the Rous virus, contains ribonucleic acid and this is supported by the reported failure to detect any deoxyribonucleic acid in biologically active though relatively crude preparations of this agent (*20*).

Recently information has been obtained regarding the type of nucleic acid probably present in a mammalian tumor virus quite unrelated to the avian agents so far considered. DiMayorca *et al.* (*21*) succeeded in making an infectious protein-free extract of polyoma-infected tissue-culture cells and showed that the infectivity was susceptible to treatment with deoxyribonuclease. This suggests that the polyoma virus, the presumed source of the infectivity, contains nucleic acid of the deoxyribose type; for more conclusive evidence the nucleic acid would have to be extracted from purified virus rather than from virus-infected cells. However, were suitable purified virus preparations available, other methods, such as those used for the avian tumor agents, could be usefully applied.

Leaving the practical considerations, the information available regarding tumor virus composition, though scanty, raises important questions concerning the basic biological nature of such agents and the possible manner in which they bring about malignant change.

It is sometimes suggested that tumor viruses have quite a special relationship with their host cells and, in the case of the Rous virus, at any rate, speculation has been current regarding some kind of genetic interaction. It is difficult, however, to see how this could take place unless widely accepted views of the primary genetic mechanism operating in cells are to be discarded, since the Rous agent is of the ribonucleic acid type. Admittedly, it is not known whether ribonucleic acid has any ultimate control of genetic pathways, but if, as knowledge increases, a mode of interaction for tumor viruses with this kind of nucleic acid appears more feasible, the phenomenon could hardly be genetic according to the present understanding of the word, unless the units of cellular heredity were also found to contain ribo- as well as deoxyribonucleic acid. At the present time there is no evidence whatsoever in the field of tumor virology to suggest that such genetic interaction between virus and host cell takes place (*22*). Explanations as to how such an interaction might hypothetically occur in the case of the ribonucleic acid-containing Rous virus must therefore be regarded as speculation in support of speculation.

There are besides this two other theoretical ways in which tumor viruses might produce oncogenic effects, and neither of these calls for the revision of accepted principles of cell biology. In either case one must assume that tumor viruses and ordinary infective viruses behave essentially in a similar manner when they parasitize cells and replicate. The existence of such a similarity is supported by the findings on tumor virus composition reviewed previously. For, the polyoma virus provides an example of a tumor agent that probably relies on deoxyribonucleic acid for its genetic continuity, whilst the Rous and myloblastosis viruses contain ribonucleic acid, thus showing that tumor viruses can possess nucleic acid of either type just as can ordinary viruses.

The manner in which these two groups of agents differ must lie merely in the rate of their replication and its resultant pathogenicity for the cell; it could then be that the tumor viruses act as such simply because during their relatively slow replication process they cause some sublethal damage to the cell which leads to its unfettered multiplication. According to one view this might be by the destruction of the ability to register the presence of outside stimuli which normally act as a check on the systems serving cell division, these systems then being allowed free rein. According to the other theory, the process of replicating a tumor virus could so pervert and damage the finely balanced normal interactions within the cell, that some enzyme system might be released from control or actually stimulated and thus bring about unlimited cell division where such should not occur.

According to either of these theories, a situation is envisaged in which the old idea that tumor viruses merely trigger off malignant change as a side effect during replication would, after all, be justified.

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PLATE

Plate 6

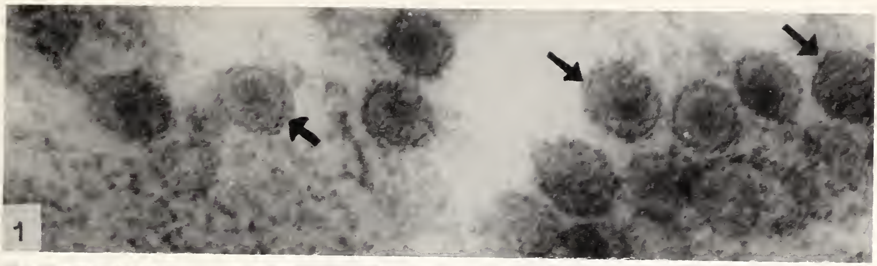
All the figures are electron micographs of sections cut through permanganate-fixed samples from the virus-containing zones of pellets prepared from fluorocarbon-treated Rous virus suspensions.

Figure 1.—Group of particles about 75 mμ in diameter showing electron dense nucleoid and less dense outer zone or viroplasm around it. The double nature of outer-limiting membrane is evident in particles most favorably orientated to plane of section (*arrows*). [Reprinted from the *British Journal of Cancer;* Epstein (*13*).] × 150,000

Figure 2.—Survey picture of control sample which had been incubated for 2 hours at 37° C. in 30 percent alcohol. The spherical virus particles appear ragged and somewhat extracted; the outer-limiting membranes and dense nucleoids are intact. [Reprinted from the *British Journal of Cancer;* Epstein and Holt (*16*).] × 70,000

Figure 3.—Survey picture of sample incubated as that shown in figure 2 except that the alcohol contained ribonuclease. The nucleoid has been removed from each particle, whereas the viroplasm and outer-limiting membranes have not been affected. Particles appear slightly swollen as if some central bracing structure has been removed. [Reprinted from the *British Journal of Cancer;* Epstein and Holt (*16*).] × 70,000

Figure 4.—Low power survey picture showing composition of virus-containing zones of pellets. Uniform particles about 75 mμ in diameter are almost the only formed structures present. [Reprinted from the *British Journal of Cancer;* Epstein (*13*).] × 14,000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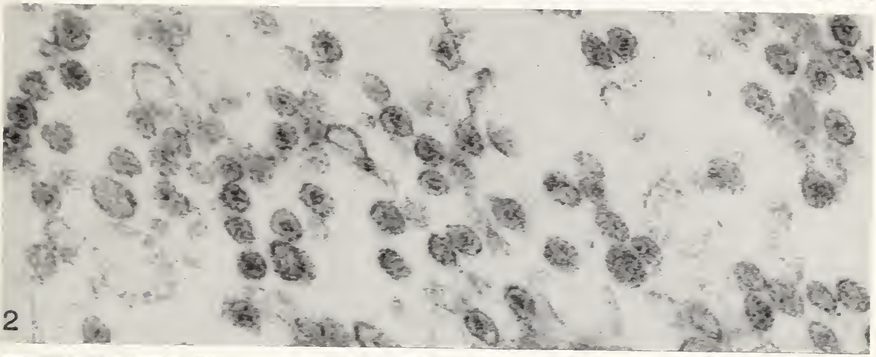
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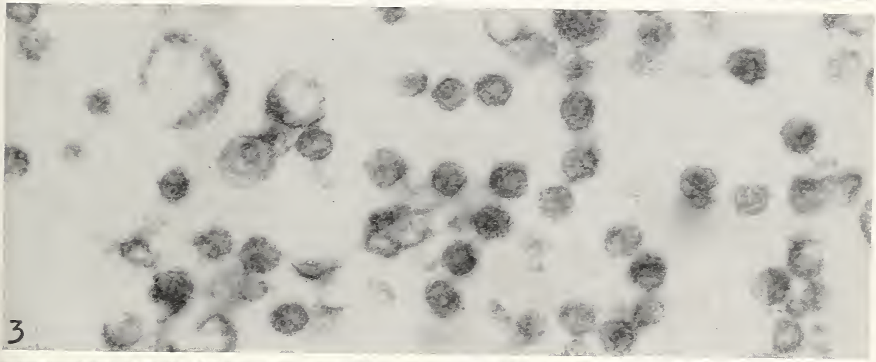
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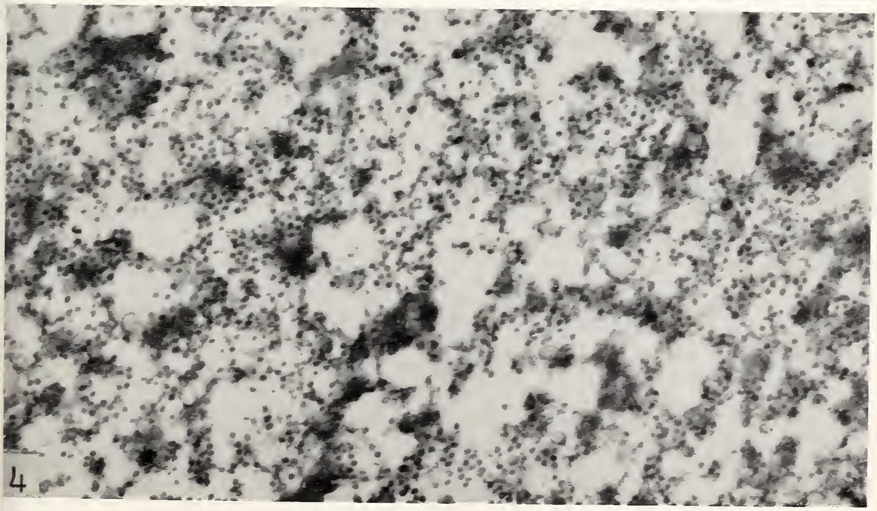
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EPSTEIN

Discussion

Dr. Mellors (Hospital for Special Surgery, New York): I wish to compliment Dr. Epstein on his fine presentation. He has wisely extended his work from an RNA-virus such as Rous sarcoma virus to a DNA-virus such as adenovirus. In considering the contents of his submitted report, I was a little concerned about the purity of the virus-containing pellets that are obtained by the fluorocarbon and high-speed centrifugation method, wondering, for example, in the case of an RNA-virus pellet whether cytoplasmic particulates containing RNA might constitute a significant contaminant.

Dr. Epstein's work was done with such thoroughness that I was sure he had an adequate explanation for this, but the extension of the study into the DNA category with such clear-cut results puts all concern to rest.

I would like to ask him, however, if he has any idea as to why acridine orange stains and fluoresces in a certain color upon combination with DNA and in another color on combination with RNA. Is this just simply a matter of the concentration of the fluoro at a particular locus, a kind of metachromatic situation? Finally, you did indicate some problems in nonspecificity even in the staining of adenovirus. You overcame this problem effectively and properly but I would like to have a little clarification as to the nature of this problem.

Dr. Epstein: Well, I am no chemist, so I do not think I am going to be able to help you very much. With regard to the first question about the specificity of this staining reaction, the finding of acridine orange and its properties came as the culmination of an immense amount of work with various kinds of fluorochromes. The first indication of the specificity of the dye for nucleic acids was given in 1956, independently in my country by Dr. Armstrong, and in this country by Dr. von Bertalanffy working with Dr. Bickis. The way their two papers appeared almost simultaneously with identical results and the subsequent work which has been done with the technique make it quite clear that for some reason which may be known to the chemists but which is not known to me, this fluorescence response is absolutely specific for the two types of nucleic acid, if you confirm it with a nuclease digestion.

If you can abolish the fluorescence with a nuclease digestion, the specificity is sound. Why occasionally—and this is the second question—you should get a nonspecific fluorescence we do not know. We think it is some sort of technical thing, but it can be circumvented by insistence on a nuclease control. And if you do that, you seem to be all right.

Dr. Dmochowski (M. D. Anderson Hospital): I will first mention the question of acridine orange which Dr. Mellors has raised. I think, Dr. Epstein, when we speak of the specificity of staining with acridine orange we have to consider two types of specificity: (1) the specificity for DNA or RNA of the host, and (2) the specificity for DNA or RNA of the virus.

Dr. Niven and Dr. Armstrong in England and Dr. Tenenbaum in this country have pointed out that in viral infections you may get a certain degree of acridine orange staining which will be either for DNA or RNA, but this does not necessarily mean that it is the viral DNA. Whether it is of viral or host origin can be tested with comparative ease by pretreatment with chymotrypsin or pepsin followed by DNase.

Staining with acridine orange for DNA may be only host response, and in this case it can be removed by digestion with DNase. If it is due to the virus itself, it cannot be removed by DNase alone, but only after treatment with pepsin or chymotrypsin followed by DNase digestion.

I would like to ask Dr. Epstein about the sections of adenoviruses. How many times did you apply the fluorocarbon treatment to the adenovirus preparations before you obtained such nice sections? Are these sections of the whole pellet or of the so-called outer zone of the pellet, *i.e.*, the upper zone of the sedimented pellet?

Dr. Epstein: Now, let us take those in order. The first one, I did not express any opinion at all as to whether the acridine orange was showing us that there was viral DNA or RNA—viral nucleic acid or host cell nucleic acid. All I said was that it is specific for nucleic acid.

The problem of what nucleic acid you are looking at is a completely different one and takes us into the field of virus isolation and purification.

The question of applying various other enzymes beforehand to test whether there is a viral or host cell nucleic acid applies only when you are looking at viruses inside a cell. It does not apply in the kind of material which we have examined because we have spun down purified virus particles, but whether there is, in addition, contaminating host cell material is another matter. If the material stained with acridine orange is checked and looked at in the electron microscope, morphological information can be obtained as to the degree of purification that has been achieved. This does not tell you whether other substances are present which cannot be seen with the electron microscope. But we think that, taken in conjunction with the electron microscope changes that are seen inside the virus when the digestion is carried out, we are justified in concluding that the material which we stained consists of virus particles.

You asked how many times we had treated the active virus with fluorocarbon, and the answer is three.

Another question was, Did this section represent the whole of the pellet? No, I described what we looked at as the virus-containing zone. There are other substances that are not removed by fluorocarbon and which come down in the pellet. However, if a Spinco centrifuge with an angle head is used, the various deposits separate out in different regions of the pellet, and an area made up of particles is seen.

That is a very extensive area and the uniformity which I showed you is not just an example of a good part; it is what is seen right through.

Dr. Kaplan: It seems to me that Dr. Colter and Dr. Epstein have made opposing statements on one very interesting point, namely, the question of cytoplasmic versus nuclear locus of action. Dr. Colter, I believe, made the statement in connection with his discussion of the Stent hypothesis that all available evidence concerning replication of viral RNP indicates that it occurs in the nucleus. I was interested in this statement, since in a review which I prepared (Cancer Res. 19: 791, 1959) I searched very hard for any evidence as to the locus of virus nucleoprotein replication, and failed to find critical evidence on this point.

Contrariwise, Dr. Epstein has come to the same conclusion that I was inclined to approach, *i.e.*, that at least with the RNA-containing viruses there is perhaps more reason to believe that their locus of action is cytoplasmic, and that if they are to be regarded as auxiliary genetic determinants of the cell, they do so by acting somewhere in the cytoplasm.

Dr. Duryee (George Washington University): It is important to think about Dr. Kaplan's remarks and his cogent analysis of the critical problem of where a tumor virus operates. In view of Dr. Epstein's concluding statement that tumor viruses operate by causing "a cytoplasmic change in the host cell purely incidental to virus replication," we need to have a better rationale. I wish, therefore, to make some general comments on Dr. Epstein's and Dr. Colter's papers.

In the frog renal adenocarcinoma, which Dr. Lucké studied for many years, we now know the locus for tumor-virus action. In spontaneous "transforming" tubules, and in tumor-filtrate injected kidneys, the first observable change is appearance of "new" DNA around the nucleolus where it attaches to its chromosome. By all criteria this attachment point is a gene locus.

The second event that happens is an increase in the RNA content of the nucleolus. It is a striking phenomenon, one which is being noted by other investigators. In human gynecological tumors it has been beautifully confirmed by Dr. Margaret E. Long and Dr. H. C. Taylor, Jr., when they measured up to 70 times the RNA in nucleoli of Grade III cancers compared to Grade I.

The third sequential event is the extrusion or appearance of RNA in the cytoplasm in the form of inclusion bodies. Thus nuclear RNA transfers to the cytoplasm to accelerate protein synthesis and to stimulate cell growth. We find that while the amount of RNA or RNP varies from cell to cell and tumor to tumor, it always correlates in amount with the degree of malignancy. Perhaps of prime importance is the heretofore unnoticed fact that DNA-aggregates may escape from the nucleus along with RNP. We have identified this DNA in amphibia as tumor virus.

One cannot escape from the observation that parallel changes in experimental frog tumors are found in a wide variety of human neoplasms, always correlating with degree of malignancy. (Duryee, Long, Taylor, McKelway, Ehrmann, 1960; Page, Regan, and McCarty, 1938.)

Finally these changes precede and lead to initiation of mitosis and eventual metaplasia or even neoplasia. In brief, nucleolar stimulation is the trigger, we believe, for all hyperplasia, although to carry out Dr. Beard's thought, it is necessary to do as we have done—prove metastasis and invasion before one can claim to have produced actual malignancy.

Thus there is invariably a spectrum of cell changes which may result from a tumor-virus infection. This question should be pondered by all of us: Do the viruses, whether they are RNA-type or DNA-type, initiate similar sequences in every host cell? In other words, let us be more diligent in examining host cell changes in their mitotic trigger mechanism. It may be worth stating again that viral infections may act simply as another carcinogen, *e.g.*, just as methylcholanthrene or intense X irradiation may act. We cannot solve the problem of cancer by direct study of methylcholanthrene; neither can we solve it by merely studying the nature of viruses. We are going to have to look more often at the point of host cell response at the DNA-gene locus where RNP is first manufactured.

Dr. George Weber (Indiana University): I would like to mention our recent studies with Dr. Levine and Dr. Ashmore on cytoplasmic enzymes of Rous sarcoma and the chorio-allantoic membrane. These studies may be of interest because of the RNA nature of the Rous virus. It was shown that the enzyme, phosphohexose-isomerase, which channels glucose-6-phosphate into glycolysis is markedly increased. A similar elevation was noted in the lactic dehydrogenase activity. These increases amounting to 300 to 400 percent are calculated on per average cell basis. This is an important consideration because the Rous sarcoma contains twice as many cells per wet weight as the chorio-allantoic membrane.

In addition to these increased activities of phosphohexose-isomerase and lactic dehydrogenase, the presence of other glycolytic enzymes was also demonstrated in this tumor (fructose-1, 6-diphosphatase and phosphoglucomutase). The shunt enzymes were also present. The increased glycolytic enzyme activities correlated well with results obtained on incubation of tissue slices with differentially labeled glucose. Both enzymatic and isotope data indicate the operation of an active shunt and increased glycolysis. It may be worthwhile to mention that similar enzymatic and metabolic behavior was found when Rous sarcomas were grown in the wing web of chickens. (Weber, G., Banerjee, G., Levine, A. S., and Ashmore, J.: Metabolic studies on the Rous sarcoma. Proc. Am. Assoc. Cancer Res. 3: 161, 1960.)

Dr. Pollard (University of Texas): The mechanism of cell-virus interaction constitutes a fundamental process which is more significant than any other single facet of virology.

Our group has been studying cell response to psittacosis virus infection by means of acridine orange stain and ultraviolet microscopy. With this procedure, RNA of the cytoplasm and of the nucleolus stains red and the DNA of the nucleus stains green-yellow. Psittacosis virus invades the cytoplasm and within hours after exposure the DNA virus particle is surrounded by a red-staining, RNase-sensitive "matrix." Within the following 48 hours the RNA-staining masses change color from red to orange to yellow-green, and at the latter stage mature virus particles become visible. This exemplifies a procedure whereby cellular response to viral activity can be studied visually. The histochemical observations indicate that the DNA of psittacosis virus initiates a mobilization of RNA by the host cell and that as the virus particles mature this transforms to DNA. Thus an intracellular indicator system becomes available through the use of fluorochrome techniques, which may have application in problems not directly associated with this particular virus.

Dr. Mellors: For some time Dr. Monroe and I have been studying Rous sarcoma in chicken wing web and in the chorio-allantoic membrane of the chick embryo with fluorescent antibodies. After Dr. Stanley's comment about the immunologists being left behind, I am hesitant to mention this procedure, but the findings on Rous sarcoma cells were interesting in this respect: Various forms of cytoplasmic particulates containing Rous viral antigen were readily demonstrated, but it was also clear that perhaps about one in a thousand sarcoma cells contained nuclear viral antigen, *i.e.*, Rous viral antigen was demonstrable in the nucleus of perhaps one in a thousand sarcoma cells as a discrete focus which sometimes lies, or, as one focuses up and down, appears to lie near the nucleolus. This apple-green focus of viral antigen may be seen above or next to a blue-violet autofluorescent nucleolar image, so that I believe, as far as the protein moiety of Rous virus is concerned, there certainly is a time when viral protein occurs in the nuclei of sarcoma cells.

Work on the cellular formation of other types of proteins, for example, rheumatoid factor or 19S γ-globulin which is formed in plasma cells, has also shown that on occasion protein antigen can be detected in the nucleus as well as in the cytoplasm of cells that synthesize these proteins.

Dr. Bryan: Dr. Malmgren and Dr. Fink have a paper in press in which they confirm the observation which you have described, that in the rare cell fluorescence can be seen in an area which appears to be the nucleolus.

Cell Sensitivity and Specificity of Response to Polyoma Virus [1]

CLYDE J. DAWE,[2] *Laboratory of Pathology, National Cancer Institute,[3] Bethesda, Maryland*

The basic nature of viral selectivity for certain species, certain organs, certain cells, and certain sites within these cells constitutes a broad and deep problem that penetrates to the level of the biochemical composition and interaction of the parts of host cells as well as of viruses. The problem is not special or new to students of tumor viruses, for from the beginning it has attracted and repelled, stimulated and discouraged, and confused and bemused investigators of virus diseases.

Logically, it has been supposed that the specificity of viruses and of response to them is in some way related not only to the genetic, but also to the epigenetic, or differentiative controls of cells. One naturally looks to experimental embryology for help, but so far it has proved that material entities concerned with morphogenesis and differentiation in higher animals have been more difficult to isolate, preserve, and analyze than have viruses. Julian Huxley (*1*) recognized the situation as recently as 1958: "The method by which tissues and organs differentiate in the course of normal development is at the moment the main blank space in biology's map."

As it seems to be a general principle in scientific advance that a field with a higher head of pressure transfers some of its potential energy to one with a lower head (provided the water gate is open), it should follow that virology currently may be in a position to increase the depth of the morphologic "mudhole." Burnet (*2*) was alert to this circumstance when he wrote that ". . . it is less widely recognized that the behavior of viruses may be equally relevant to the understanding of the differentiation of parts and morphologic control within the cell." One of the purposes of this presentation is to propose that the converse may also be true.

It may seem strange that the polyoma-tumor complex should be selected as a subject for studies dealing with sensitivity and specificity of response. Of all presently known tumor viruses, this agent appears to be the least discriminated against by the largest variety of specialized cells, at least in the mouse. It should be noted parenthetically that, although the term polyoma has found wide usage, the word itself lacks a certain amount of specificity, for before this agent was recognized it was established by investigators of avian leukoses (*3, 4*) that the viral agents of these diseases can induce multiple types of tumors, and might be referred to as polyvalent. It will become apparent during the course of this presentation that the polyoma virus, like other viruses, shows definite signs of limitations of action in many parameters, including those relating to species susceptibility, strain susceptibility, tissue susceptibility, and type of response by different cells as well as by cells of the same morphogenetic lineage at different levels of development. Because they offer breadth in variety of types and location of cell-virus interaction while yet retaining specific limitations, the several polyoma virus-tumor systems represent a versatile set of probes, usable for exploration of many facets of tumor-virus phenomena.

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[1] Presented at a Symposium on Phenomena of the Tumor Viruses, New York, N.Y., March 25 and 26, 1960.

[2] The author is grateful to Mr. Willie D. Morgan, Mr. Robert Velthuis, and Mr. Edward J. Soban for technical assistance, and to Mr. Gebhard Gsell for the photographs.

[3] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

I. GENUS, SPECIES, AND STRAIN SPECIFICITY

Tumor viruses as well as infectious disease viruses, although more or less specific for certain tissues in a given species, may have a different pattern of tissue selectivity in another host species. For example, herpes simplex virus is predominantly dermotropic in man, but relatively more neurotropic in rabbits. Viruses of the Coxsackie group are another example. The polyoma virus is perhaps the most remarkable among the tumor viruses, since it produces tumors of the salivary epithelium, mammary epithelium, hair-follicle epithelium, thymic epithelium, and in several other sites in the mouse, including some connective tissues (*5*). In the rat, only connective-tissue tumors are produced (*6*). Such species differences suggest an analogy with morphologic control mechanisms recognized in experimental embryology. For example, in the grass frog (*Rana fusca*) the eye vesicle is necessary for induction of lens development from the overlying epidermis (*7*). In the water frog (*Rana esculenta*) the lens will develop in the absence of the influence of the eye vesicle [*see* Grobstein (*8*) for discussion of time and quantitative factors]. Furthermore, "strange" ectoderm from the belly of the water frog fails to form lens in response to eye vesicles that do induce lens either in lens-forming or belly-ectoderm epithelium of *R. fusca* (*9*). From this it would appear that lens epithelium, although known to be similar antigenically over a wide species range, as well as ectodermal epithelium in other regions of *R. fusca*, is biologically a different tissue from comparable epithelium of certain other frogs, and it might be expected to respond differently to a single hypothetical virus.

A similar example is to be found in the induction of balancers in larvae of newts and salamanders. Larvae (axolotls) of the salamander *Amblystoma tigrinum* do not have organs known as balancers, whereas larvae of the newts (Triton) do have them. However, it was shown by Mangold (*10*) that the proper area in the head of the axolotl is capable of inducing balancers in grafted belly ectoderm of the newt. Thus a certain area of ectoderm from rather closely related species responds differently to a given morphogenetic influence. If a virus or any other tumor-inducing agent were to be cast in the role of the morphogenetic influence, the situation would be analogous to positive and negative reactivity to that agent by homologous tissue of different species. A third example illustrates a slightly different type of analogy. A specific region in the mouth endoderm of the urodeles has been shown to be responsible for the development of teeth. When the prospective tooth-developing primordium of the urodeles is substituted by the same region from anurans, a horny type of jaw characteristic of the anurans develops instead of teeth (*11*). Here, homologous areas of ectoderm respond to the same morphogenetic influence, but in a different way. Experimental embryologists (*12*) have referred to an "intrinsic latent repertoire of potencies" characteristic of each species, and ascribe this repertoire to limitations placed upon cell reactivity by genetic factors. Analogous situations for virus infection in mammals are difficult to find, possibly because in animals infected at birth or later, the variety of ways in which most cells can react has already been realized with reference to differentiative direction. Perhaps an example is to be found in the mixed tumors of fat and bone (*13*, *14*) in mice receiving polyoma virus. Such tumor response with bivectoral differentiative capacity has not yet been reported in rats or other animals infected with this agent, though simple bone tumors of the skull in rats have been noted by Fogel and Sachs (*15*).

Adding to these illustrations some experimental evidence for a contact infection-like behavior of certain developmental organizers (*16–18*) [*see also* Grobstein on viroid transmission of inducers (*8*)], we are provided with enough resemblances between virus systems and developmental induction systems to warrant a search for possible interlocking areas.

Before one can take advantage of species differences in devising experiments to investigate possible relationships between morphogenetic and differentiative influences and viral response, it is necessary to have information regarding each of these two systems centered about the same tissue. How, then, do different species respond to polyoma virus?

The pitfalls introduced by dose-response effects and viral strain differences must always be kept in mind when this question is answered. It is regrettable that there is not available a carefully quantitated study to answer it fully. At the all-or-none response level, however, it was established in the act of discovery of this agent (*19–21*) that mice respond to it with tumor development in the salivary glands, adrenal glands, and subcutaneous connective tissue. Later Eddy *et al.* (*22*) reported an oncogenic response by golden hamsters, and then by rats (*6*). Eddy and Stewart (*23–25*) next reported the development of tumors, followed by regression, in rabbits. More recently, Rabson *et al.* (*26*) have found that the multimammate African rodent, *Rattus* (Mastomys) *natalensis*, also develops tumors in response to polyoma virus. This is the sum of present positive knowledge on species susceptibility *in vivo*. Numerous reports confirm the tumor-inducing activity of the virus in mice, hamsters, and rats, and only a few are cited here (*13, 15, 27–29*).

Negative data are of almost equal importance. Rowe (*30*) has failed to find serologic evidence of polyoma-virus infection in man, and isolation of this virus from man or from human tumors has not been positively demonstrated. Rowe (*30*) has also found opossums, porcupines, chickens, dogs, and other wild as well as domestic animals to be free of virus, and to have negative serologic reactions. Although Gross (*31*) mentioned induction of parotid tumors in a few mice receiving extracts of normal guinea-pig tissues, guinea pigs have not been reported to develop tumors in response to polyoma virus. Fogel and Sachs (*15*) reported no tumor response by guinea pigs, chickens, and dogs after observation period up to 4 months. Eddy and Stewart (*32*) are observing monkeys inoculated with polyoma virus, but thus far have not noted tumors attributable to the effects of this agent.

Separate attention must be given to susceptibility of tissues of different species to the virus in tissue-culture systems. In monolayer cultures, Stewart and Eddy were first to report propagation of polyoma virus in monkey-kidney cells (*33*) and then in mouse-embryo cultures (*22*). Cytopathogenic effects were not noted by them in these reports, but were later reported in trypsinized, monolayer cultures of whole mouse embryos (*34*). Rabson and Legallais (*27*) observed viral multiplication and cytopathogenic effect on milk-medium cultures of an altered murine lymphoma cell line, P388 D_1. Dawe, Law, and Dunn (*13*) reported similar findings, using the same cell line grown in a human serum medium, and reported cytopathogenic effects in two other hemic tumor cell lines of mice, P329 (*35*) and the MBIII cell line of De Bruyn (*36*). It has also been found possible to establish carrier cultures of P388 D_1 cells infected with the agent (*13*). Dmochowski (*37*) reported growth of a hamster-adapted strain of polyoma virus on human amnion cells as well as on rat-embryo cells. Dulbecco (*38*) recently noted a proliferative response by infected cultures of hamster cells, not accompanied by any cytolytic response.

Morphologic observations on the effects of polyoma virus upon intact fragments of salivary-gland tissue of the newborn mouse *in vitro* have been described by Dawe and Law (*39*), who found proliferative as well as cytocidal effects side by side in the same cultures. These effects were noted as early as 14 days after exposure of the tissues to virus, but were more pronounced after periods of 19 to 48 days. This experiment has subsequently been repeated in an identical manner, with the same virus pool and the same gelatin sponge matrix method of culture but substituting human fetal tissues for the salivary-gland tissue of newborn mice (*40*). Two fetuses serving as tissue donors were at about the 6th and 8th months of development. The tissues used were from the parotid, submaxillary, thyroid, and thymus glands. A total of 18 infected and 18 control cultures was studied by serial sections after periods up to 30 days of incubation. Survival of the salivary glands was good, but no recognizable cell response, either cytolytic or proliferative, was observed. For reasons to appear later in this paper, more extensive trials with human tissues should be made with this and other systems. Similar tests for reactivity of salivary glands of the rat, hamster, and Mastomys to polyoma virus are in progress in this laboratory.

Information on possible differences among different species of mice is lacking. Rowe (*30*) found that "wild" mice (*Mus musculus*) trapped from certain geographic areas showed positive serologic reactions against polyoma virus, whereas wild mice from other areas were serologically negative. The responsiveness of wild mice in terms of tumor development is not known.

Information as to host-strain differences of susceptibility is limited entirely to the mouse. This area requires further exploration, because recognition of distinct strain differences in susceptibility would offer opportunity for analysis of genetic influences. The mechanism of action of genetic influences could then be sought in differences of immune response, differences in timing of embryologic development, differences in endocrine factors, or constitutional intracellular differences of the host cells concerned.

Thus far, every inbred strain of laboratory mouse adequately tested has been shown capable of responding to polyoma virus with tumor development in at least one organ, the parotid gland. Positively responding strains include: AKR (*13, 41–43*), C3H/An (*44*), C3Hf/He (*5, 45*), C3Hf/Lw (*13, 45*), C3H/BiGs (*19*), C3H/BiLw (*45–47*), C3H/Fg (*13*), C58/Lw (*13*), C57BL/Ka (*13*), C57BL/Ha (*42*), C57BR/Lw (*13*), DBA/1 (*42*), DBA/2 (*13*), RFM (*13, 48*), StOLI/Lw (*13*), SWR (*13*), Swiss/IcrHa (*42*). In addition to these inbred strains, noninbred Swiss mice and hybrid (C3H/f × AKR)F_1 mice were among the first to be shown responsive to this agent (*5, 21*). The (AKR × Swiss)F_1 cross also responds (*42*) as do (C3Hf/Lw × AKR)F_2 mice (*49*).

At least three strains have given evidence of partial resistance: C57BL/Ka (*13*), C3H/Lw (*44, 45*), and C57BR (*13, 50*). In an early report of Gross (*50*), no tumor response was observed in strain C57BR. However, when highly active tissue-culture preparations of virus were used (*13*) it was found that one of 6 C57BR animals developed parotid tumors after receiving a dose that produced tumors in more than 90 percent of mice of other strains. The apparent relative resistance of this strain has not been further studied. Preliminary evidence on the relative resistance of C57BL/Ka mice has been presented (*13*) and is included in table 1. Not shown in the table is that among the 3 C57BL/Ka animals that developed tumors, 1 had unilateral parotid tumors only, 1 had bilateral parotid tumors only, and the 3d had a thymic tumor and bilateral parotid tumors only. It was of further interest that the parotid tumors in all 3 were unusually uniform histologically, all having a solid, "medullary" pattern without glandlike structures or the pleomorphism generally found in these tumors. Among the 39 tumor-bearing mice of other strains, there were 27 with thymic tumors, 17 with small thyroid tumors, 12 with bone tumors, 7 with hair-follicle tumors, and 5 with mammary tumors.

TABLE 1.—Tumor response to polyoma virus* by strain C57BL/Ka as compared with 5 other inbred lines

Strain	Number of mice	Number of mice surviving 1 month	Number of mice with parotid tumor	Number of litters (*positive/total*)	Latent period (*months*)
DBA/2Lw	16	11	11	2/2	2.9
STOLI	14	4	3	1/1	3.7
AKR/Lw	21	10	10	2/2	1.4
RFM	14	13	11	2/2	1.9
C58/Lw	5	5	4	1/1	2.5
Total for strains other than C57BL/Ka	70	43	39 (91%)	8/8	—
C57BL/Ka	91	41	3 (7%)	2/7	3.5

*All animals received 0.05 ml. of culture-fluid pool from first passage on P388 D_1 cells, diluted 100 :1, when less than 24 hours old.

Plaque techniques have been applied to the polyoma virus (*51–54*) and the results of two of these studies (*52*, *53*) indicate that only a single agent is concerned in the induction of multiple tumor types. On these grounds, it is evident that relative resistance may be manifested by quantal response differences, by a smaller variety of tumor types induced, and by an increased latent period required for the appearance of tumor. This renders the evaluation of dose-response effects of polyoma virus at once immensely interesting and mathematically intricate. The system is probably the most complex one available for application of the principles reviewed by Bryan (*55*) for the quantitation of tumor viruses. As yet an ideal study correlating particle counts, hemagglutination titer, mouse infectivity, tissue-culture infectivity, and oncogenic response has not been reported. A positive correlation between particle counts and hemagglutination titer has been found by Kahler *et al.* (*56*) for one strain of the agent.

Tissue and Cell Response

It has already been indicated that the question of sensitivity and specificity of cell response is inseparably bound to the problems of dose response, virus-strain homogeneity, and genetic character of the test animal. Historically, it will be recalled (*19–21*) that the variety of tumors observed in mice, after inoculation with direct extracts of leukemic tissues, was much smaller than that seen subsequent to passage of the agent through tissue cultures, either of monkey kidney (*33*), normal mouse embryo (*5*), or of mouse lymphoma cells (*13*). Only parotid tumors, subcutaneous fibrosarcomas, adrenal medullary tumors, and occasional thymic tumors were observed before tissue-culture passage. After receiving culture-passaged material, mice developed a much wider variety of tumors and other lesions, reported by Stewart, Eddy, and Borgese (*5*) to be as high as 23. That this change of response was due to a dose-response effect of a single virus is supported by Buffet and associates (*57*). The production of a similar effect, through partial purification of virus by differential centrifugation, and by viral cloning studies by plaque methods, was reported by Eddy and Stewart (*51*), Stewart *et al.* (*52*), and Sachs *et al.* (*53*). Possible objections to the plaque method for cloning this agent in mouse-embryo cultures have been raised (*39*), but at present there is no compelling positive evidence that more than one agent is concerned in the induction of tumors of salivary glands, lacrimal glands, submucosal glands of the upper respiratory tract, thymus gland, mammary gland, hair follicles, thyroid gland, tooth-germ epithelium, adrenal gland, renal medulla, and bone. Others (*13*, *42*, *48*) have confirmed this finding, by and large, but it has also been stressed (*13*, *14*) that the polyoma virus by no means possesses "cancer pan-tropism" (*37*). This point deserves re-emphasis, since it has been the author's experience that many who are not actively working in this field seem to have developed the impression that polyoma virus can induce tumors in *any* organ, and conversely, that any tumor of the laboratory mouse should be considered a "polyoma tumor" until proved otherwise. This attitude is not in accord with the known facts. The majority of the tumors induced by this agent have special histologic and biologic characteristics in which they differ from previously known murine tumors, even of the same organ. Dunn (*see 21*) noted the unique histologic character of the salivary-gland tumors, and also delineated certain features of mammary tumors associated with the polyoma virus in which they differed from mammary tumors arising in mice with the milk agent (*13*). It is noteworthy that the tumors induced or accelerated in mammary tissue by 2 different viruses (Bittner milk agent and polyoma virus) have distinct histologic patterns, as well as differences in distribution between sexes. This suggests that the two agents operate through different pathways, or enter the same pathway at different points. A similar situation may perhaps exist for virus-induced leukemias and leukemia-like conditions, though it is not certain that these agents all act initially on the same cell type.

It may be remembered that only a decade ago it was often said by investigators, wearied of histologic classifications, that when causative agents of tumors were found, these old confusing classifications could be dispensed with. It is reassuring to microscopists that, now that some causative agents are known, there seems to be more need than ever to classify histologically the corresponding tumors. It is imperative to be aware, however, that most of our evidence today indicates that in the polyoma virus we are dealing not with a new-found cause of a previously familiar disease, but with a newly found disease and a newly found agent causally related to it. This awareness in no way detracts from the system as a research tool, but is indispensable for proper use of the tool and understanding its products.

Differences in classification already exist with respect to many of the tumors associated with this agent. Some cases in point will be discussed here because they are important to the interpretation of past reports as well as of future investigations.

The tumors of the thymus gland have now been described by many investigators (*5, 13, 14, 48*). They were designated thymic epitheliomas by Stewart (*5*) who was first to recognize them as part of the polyoma pattern. Later, Buffet *et al.* (*57*) described similar tumors in mice inoculated with polyoma virus, but classified them as reticulum-cell sarcomas. McCulloch *et al.* (*58*), working with an agent apparently closely related to, if not identical with polyoma virus, also observed thymic tumors and designated them as thymic lymphomas. It will readily be seen that baffling complications are brought into the picture if the thymic tumors are either lymphomas or reticulum-cell sarcomas. For, in the early stages of recognition of the polyoma virus, there was considerable question as to whether this agent and the leukemia agent of Gross were one and the same (*59*). If the thymic tumors following polyoma-virus inoculation are neoplasms of hemic cells, the possibility of either a genetic relationship to, or an admixture with the leukemia agent of Gross would have to be considered. Morphologic evidence for the epithelial nature of the tumors has been put forth by Stewart *et al.* (*5*) and by others (*13, 14*). There is also biologic evidence for this view. Transplantation attempts carried out by Law (*49*) have resulted in successful transfer of thymic tumors to only 2 of approximately 200 recipients of compatible strain. These 2 successful transfers occurred in 2 animals of the same litter, inoculated with a cell suspension when less than 24 hours old. The transplanted tumors appeared after a latent period of nearly 3 months at the site of subcutaneous inoculation. No tumors of salivary glands or other organs were found, and the histology of the transplants was identical with that of the primary tumor, including the presence of large numbers of lymphoid cells adjacent to the proliferating epithelial masses. There was no evidence of leukemia or lymphoma in any organ of these animals. The extreme difficulty of transplantation and the failure to yield a morphologic picture consistent with a lymphoma or leukemia are strong evidence that these tumors are not hemic in nature.

Perhaps closely related to the problem of the thymic tumors is a situation that is found only occasionally in the parotid-gland tumors. Among the multicentric tumors of the parotid gland there are some examples that resemble, but are not identical with, the thymic tumors, and are also not tumors of ectopic thymus, such as have been described (*13*). Yet they contain relatively large numbers of lymphoid cells, and relatively small numbers of epithelial cells (figs. 1 and 2). The epithelium is confined to a few tubular structures, or may be distributed perivascularly, as in the thymic epithelial tumors. These tumors might be mistaken for lymph nodes or lymphocytic neoplasms, since they are composed predominantly of lymphoid tissue. Recognition of the epithelial component reveals their compound nature. On close examination, it becomes evident (fig. 2) that the lymphocytes in these tumors are aggregated in groups in which all stages of maturation are represented. This is unlike the situation in lymphocytic neoplasms, where uniformity in the degree of maturation, or lack of it, is the rule. Dr. M. H. Salaman, on a visit to our laboratory, informed us that he has been investigating this type of tumor in transplants and was kind enough to show and give us sections. Two tumors transplanted through several generations repeatedly showed the compound "lymphoepithelial" pattern described previously, and none of them resembled usual lymphocytic neoplasms.

The evidence here presented indicates that this special type of parotid tumor is both histologically and biologically similar to the thymic epithelial tumors. This is not surprising, if one considers that the parotid gland and thymus originate embryologically in proximity to one another in the primitive pharynx. In humans afflicted with Mikulicz's disease, the parotid gland attains huge proportions as a result of lymphoid accumulations. The term "benign lymphoepithelioma" has been applied to these enlargements (*60*). It is suggested, therefore, that the similarity between thymic tumors and the lymphoepithelial tumors of the parotid gland in mice is based on an active or a potential affinity for lymphoid cells, acquired by the thymus and parotid gland in the course of embryogenesis. This potentiality is realized under normal conditions in the thymus gland, but only under exceptional and poorly understood circumstances in the parotid gland.

Inclusion of the hair-follicle tumors under the heading of epidermoid carcinomas (*5*) does not appear valid. The histology of these tumors has been studied closely (*13, 14*) and it is certain that the invasiveness and the cellular characteristics of squamous carcinoma are lacking. These tumors cannot be placed in any previously established category. We have not seen metastasis, the origin of which could be traced to this lesion, among more than 100 animals bearing literally thousands of individual tumors of this type. These tumors should not be accepted as the equivalent of squamous carcinoma in any studies of pathogenesis.

The same restraints apply to the epithelial tumors involving the mandible and maxilla. The morphologic resemblance of some of these tumors to ameloblastomas has been previously noted (*13*), and their possible origin from epithelium of the dental lamina deserves further investigation. Squamitization occurs within some of these tumors, but they do not have other features of squamous carcinoma.

It is improbable that squamous carcinoma of the forestomach (*5*) is related causally to polyoma virus. We have encountered only one example of this among many hundred mice autopsied after inoculation with this agent (*61*). Histologically this example was similar to "spontaneous" carcinomas that occasionally occur at this site (*62*). It was readily transplanted by subcutaneous route.

The relationship of some primary lung tumors to polyoma-virus infection is problematic. Two primary lung tumors were listed by Stewart *et al.* (*5*), and Latarjet and de Jaco (*43*) reported several in mice receiving extracts from leukemic tissue. Stewart *et al.* (*5*) and later Dawe *et al.* (*13*) noted ballooning nuclei and cellular enlargement of bronchial epithelium, but whether these lesions can and do progress to tumors has not been established. That they well might is suggested by Rabson's observation (*63*) of multiple bronchial epithelial tumors in hamsters receiving large intratracheal doses of virus.

The pleural, pericardial, and peritoneal lesions described by Stewart *et al.* probably are related causally to the polyoma virus, and offer a unique opportunity for study of mesothelial proliferations. The absence of these lesions in our material (*13*) is perhaps explained by exclusion of noninbred Swiss mice from the study.

The tumors composed of mixtures of adipose cells and bone, and the adrenal medullary tumors arising in mice receiving this agent have been previously commented upon with regard to their novel character (*13*, *14*). The origin of the adrenal tumors from medullary cells is still open to some question because of their striking deviation from the histologic pattern of "spontaneous" medullary tumors, or pheochromocytoma (*13*, *21*). They have also failed to show any functional activity.

Several other tumors have occurred too infrequently in mice inoculated with polyoma virus to warrant an opinion as to whether they were spontaneous or arose as a result of treatment. It is presently largely a matter of opinion as to whether the harderian-gland tumors (*5*, *13*), the urethral-gland tumors (*13*), the subcutaneous hemangioendotheliomas, and the hemangiomas of the liver (*5*) are specifically related to the activities of the agent.

A peculiarity of some small tumors of the extraorbital lacrimal gland of the mouse deserves brief description here because it raises some perplexing questions. This peculiarity consists of greatly enlarged cells in the acini immediately adjacent to small foci of tumor (figs. 3 and 4). Both cytoplasmic and nuclear volume are increased several fold. Moreover, mitoses in these cells are very numerous, even though the cells are clearly not part of the tumors as they are ordinarily recognized. The mitotic figures also contain numbers of chromosomes that place these cells definitely in the polyploid category (fig. 5), and frequently one or more chromosomes appear to have been separated from the spindle in a manner not unlike that seen after radiation injury. Does this phenomenon represent a different, non-neoplastic response to the virus? Is it a response not to viral infection, but to some product of cell-virus interaction released by the adjacent tumor cells [*see* Stanton (*14*) and Morgan (*64*)], or is it a response related only to nonspecific necrosis of other parenchymal cells, similar to the phenomenon described by Teir (*65*) in extraorbital lacrimal glands of rats inoculated with extracts of normal lacrimal-gland tissue?

In addition to the neoplastic response to polyoma virus, several non-neoplastic or possibly preneoplastic responses by the mouse deserve mention. These include hypertrophy and hyperplasia of epithelium of the renal tubules, the "runting phenomenon" first described by Stewart *et al.*, and the recently reported occurrence of hydrocephaly in mice inoculated intracerebrally during the suckling period (*66*). The renal tubular lesion contains elements both of necrosis and of proliferation, and has been classified as preneoplastic by Buffet *et al.* (*57*). This lesion offers a challenge to investigators interested in cocarcinogens and endocrine influences. Would a steroid influence shift the balance in the direction of neoplasia at this site, as suggested by the influence of estrogens on the development of renal tumors in hamsters (*67*)?

In the opposite direction, is it possible that a necrotic response on the part of mesenchymal cells, similar to that observed in tissue cultures, could account for retarded bone growth and the consequent runting phenomenon? Studies of bone growth in infected organ cultures, after the method of Fell (*68*), might prove helpful in answering this question. It is anticipated that the viral studies to be reported later in this symposium by Dr. Rowe (*30*), will also bear on this point. So little is known of the pathologic anatomy of the hydrocephalic phenomenon associated with this virus that it would be premature to make speculations as to mechanism at this time.

The hamster, rat, and Mastomys respond to polyoma virus in what appear (deceivingly?) to be less complex patterns. Among these animals, only one type of epithelial tumor has thus far been demonstrated, and this in the golden hamster. This finding was recently made by Rabson *et al.* (*63*) who observed multiple proliferations of the bronchial epithelium in hamsters exposed to heavy doses of virus by intratracheal insufflation. The lungs of these animals were studded with proliferating cell aggregates, apparently originating chiefly in the smaller bronchi and extending into pulmonary alveoli, where they presented an appearance similar to the pulmonary lesions of squirrels receiving the squirrel fibroma virus intratracheally (*69*). It is of special interest that occasional foci within some of the nodules also showed squamous characteristics.

Mesenchymal tumors of at least 2 types—angioid, and solid spindle-cell—have been seen in hamsters (*22, 27–29*), rats (*6, 15*), and Mastomys (*26*), after subcutaneous introduction of virus. The situation is made complex by the fact that in each of these species the anatomic distribution of the tumors is different. There are already differences of opinion as to whether the vascular lesions of the liver in hamsters represent neoplasia in even a broad definition (*70*). In the hamster, subcutaneous tumors may be either solid fibromas or fibrosarcomas, or angiosarcomas (*29*). Primary tumors in the liver and lung of hamsters have been classed as angiomas or angiosarcomas (*22*). Tumors of the heart, large vessels, kidneys, and serosa of the gut present the picture of solid sarcomas (*22*), as do intracranial tumors occurring after intracranial inoculation (*71, 72*). In the rat, tumors are not so widespread in the mesenchymal tissues. The renal tumors have either a solid or angioid spindle-cell make-up, and the subcutaneous tumors are solid fibromas or sarcomas (*6*). Those of the skull (after intracranial inoculation) are bone-forming (*15*). Mastomys responds to subcutaneous inoculation in a manner somewhat intermediate between hamster and rat, with solid fibrous tumors of the kidney, angioid tumors of the liver, and subcutaneous fibrous tumors (*26*). It is interesting that solid fibrous tumors of the kidney and subcutaneous fibrous tumors are the only types common to all known responsive animals. This suggests that mesenchyme of the renal medulla and of the subcutaneous tissues may be less "species specific" than other tissues. In this connection, it should be noted that the renal site happens to be an opportune one for investigation of possible relationships between morphogenetic influences, viral susceptibility, and type of response. The first step would be to determine if these tumors arise from the metanephric mesenchyme, as is suggested by their location at the corticomedullary junction. A technique for isolating metanephrogenic mesenchyme and epithelium of the ureteral bud has been made available by Grobstein (*73*, *74*). It is fascinating, but too early, to speculate far on the implications of Grobstein's observation (*73*) that epithelium of the salivary gland (the organ most responsive to polyoma virus in the mouse) is the only epithelium, except that of the ureteral bud, which is capable of inducing tubule formation in the metanephrogenic mesenchyme, in tissue culture. This set of circumstances may be purely coincidental, as it was shown also by Grobstein (*73*) that spinal cord, even from the chick, also has a strong inductive action on metanephrogenic mesenchyme of the mouse.

II. DEVELOPMENTAL INFLUENCES

The discussion so far has been concerned with the matter of *where* the polyoma agent acts, in terms of species, strain, and tissue specificity. It is also important to know *when* it can act, in terms of stage of morphogenesis or differentiation of the tissues, and how the response may differ depending on differences in these influences. Here the advantages of the polyoma virus-tumor system become more apparent, for at this point there exists a crossroads of three major disciplines (virology, oncology, and experimental embryology) largely surveyable by variations of a single methodology, tissue culture.

For nearly a decade, studies of virus tumors in mammals have been increasingly influenced by the findings made possible by wider use of young and, particularly, newborn animals. The ostensible reasons for the greater sensitivity of young animals to tumor-inducing viruses are (1) as for other viruses, the undifferentiated cells of young or embryonic animals are "inherently" more susceptible to virus infection, as are cells in tissue culture, and (2) the poorly developed reactivity of immune response in young animals permits viruses to disseminate, infect cells, and multiply to still higher concentrations before the defenses come fully into play.

Tissue-culture methods make it possible to control the second influence, thereby simplifying (not without pitfalls) evaluation of the first. Evidence will be presented here to show that for the action of polyoma virus on salivary-gland tissue, the first tenet deserves critical re-examination, at least with regard to the *response* elicited by virus acting on cells in different stages of pre- and post-natal development.

The observation that the oncogenic response of salivary glands in the intact mouse is more marked in the newborn period and declines progressively with increasing age remains unchallenged. The absolute limitation of the responsive period to the first postnatal day (*75*) found refutation, however, when highly active tissue-culture preparations of virus made it possible to show that salivary tumor could be induced in 50 percent of mice inoculated as late as the 14th postnatal day by the usual subcutaneous route (*13*). Parotid tumors also have appeared after long latent periods (8 months or more) in mice inoculated intravenously, when 30 to 40 days old, with massive doses of the agent (*13*). Furthermore, it became apparent that ability to respond could be restored to some degree in young adult mice by total-body irradiation prior to exposure to virus (*13*). This observation has been repeated with mice up to 5 months of age (*76*), though it has not been possible by X irradiation to restore the same degree of response seen in newborn mice, either in terms of proportion of animals developing tumors, multiplicity of tumor types appearing, or shortness of the latent period. The important single fact remains, however, that salivary glands of fully mature mice are capable of tumor response to polyoma virus.

A possible cotumorigenic action of radiation, as against an immune-response suppressing effect, has not been disproved from the foregoing observations. Use has been made, therefore, of the tissue-culture system previously reported (*39*), in which it was observed that fragments of salivary gland exposed to the virus in tissue culture responded in a dual manner, and exhibited both proliferative and necrotic response, with the former dominating during the periods studied.

The results of this study will be reported briefly here and are tabulated in table 2. It is recognized that two objections can be raised to this approach: (1) It has not yet been established that the morphologic changes observed in this tissue-culture system are biologically equivalent to tumor genesis in the intact animal. It appears probable, however, that there is a close similarity between the phenomenon *in vitro* and *in vivo*. We have recently observed the appearance of a tumor, morphologically identical with salivary-gland tumors induced *in vivo*, at the site of transplantation of a 30-day organ culture of submandibular gland infected with the polyoma virus (figs. 11, 12, and 13). More observations on such transplanted cultures will be required for careful evaluation of the nature of the changes seen *in vitro*. (2) Although the tissue-culture system provides conditions that sustain cellular survival and proliferation, these conditions do not duplicate conditions *in vivo*, either in regard to constancy of the milieu or to qualitative and quantitative nutritional factors. As with all tissue-culture studies, the value of the observations depends on how well they accord with other information, and on what leads they provide toward the design of other investigations.

Gelatin sponge matrix cultures of submandibular (submaxillary) gland from a 5-month-old C3H/Bi male mouse were the first to be studied besides the glands of the newborn. The technique was the same as that

TABLE 2.—Response to polyoma virus by submandibular glands of mice at various ages

Age of donor animal	Hemagglutination inhibition titer of donor or donor's mother	Response at indicated interval after isolation and exposure to virus				
		1 Week	2 Weeks	3 Weeks	4 Weeks	6 to 8 Weeks
13-Day embryos (complete rudiment of submandibular gland)	Not determined, mothers from polyoma-free breeding colony	± Cytolysis, mesenchyme only + Nuclear inclusions	+ Cytolysis of epithelium and mesenchyme + Nuclear inclusions	+ Cytolysis of epithelium and mesenchyme + Nuclear inclusions	—	—
14-Day embryos (complete rudiment of submandibular gland)	Not determined, mothers from polyoma-free breeding colony	—	+ Cytolysis of epithelium and mesenchyme + Nuclear inclusions	+ Cytolysis of epithelium and mesenchyme + Nuclear inclusions	+ Cytolysis of epithelium and mesenchyme + Nuclear inclusions + Epithelial proliferation (minimal)	—
Newborn, 12 hours	Negative	? No definite reaction	+ Cytolysis of epithelium and mesenchyme + Nuclear inclusions + Epithelial proliferation	+ Cytolysis of epithelium and mesenchyme + Nuclear inclusions + Epithelial proliferation	+ Cytolysis of epithelium and mesenchyme + Nuclear inclusions + Epithelial proliferation	+ Cytolysis of epithelium and mesenchyme + Nuclear inclusions + Epithelial proliferation
Young adults, 6 and 8 weeks	Negative	—	—	—	+ Cytolysis of epithelium and mesenchyme + Nuclear inclusions + Epithelial proliferation	—
Adult, 5 months	Negative	—	—	—	Same as above	—
Adult, 8 months	+ 1:800	—	—	—	Same as above + Epithelial proliferation (maximal)	—

previously used, except that it was found necessary to replace the gelatin sponges repeatedly during the first 10 days of culture because the adult male submandibular gland secretes a protease that rapidly digests the gelatin (*77*). Activity of this enzyme was not apparent after 10 days in culture.

The culture medium used throughout the work reported here was composed of human adult serum (30%) and Morgan, Morton, and Parker's medium 199 (70%).[4] It also contained 100 units per ml. of penicillin G and phenol red at a concentration of 0.001 percent.

After 30 days, the cultures were fixed, serially sectioned, and examined. Not only had the adult tissue survived well, but the response to the virus preparation was similar to that observed previously in cultures of salivary glands from newborn mice. In fact, the proliferative aspect of the response was perhaps more marked. Figure 6 shows the heaping up of large, altered cells around the periphery of an explant, and extension of groups of cells into the compartments of the adjacent gelatin sponge. The nuclear changes and cell necrosis seen in the experiments with glands from newborns were also present.

Five additional culture series were then set up, using as donor mice 4 C3H/Bi males at 6 and 8 weeks of age with negative hemagglutination inhibition (HI) serologic reactions to polyoma virus, and one C3H/Bi female at 8 months of age, with a positive HI reaction to a dilution of 1:800.[5] This last animal had never received polyoma virus by injection and bore no tumors, but was a member of a colony in which the incidence of natural infection was high. The first 4 animals had been reared and kept in a room free of polyoma-virus infection.

The general results in all these culture series were similar, though certain differences should be noted. Regardless of the age, sex, or serologic status of the donor, cytologic alterations including nuclear inclusions and clear areas, nucleolar enlargement, cell enlargement, proliferation and extension of cell groups into the sponge, as well as necrosis, were found. All these changes were similar to the response exhibited by glands of newborns. Figures 7 and 8 illustrate explants from test and control cultures, respectively, from an 8-week-old mouse. It will be noted that the control explant contains relatively well-preserved, though atrophied, remains of acinar and duct structures, and that these epithelial structures are completely quiescent and remain within the confines of the original explant. In contrast, there is a rather massive outgrowth of cells from the virus-exposed explant into the sponge. Figures 9 and 10 show the cytologic differences between the surviving cells in a control explant and the outgrowing cells from an infected culture. It is evident in figure 10 that, as in the study with glands from newborns, cell necrosis and mitosis can exist side by side among the altered cells. Another feature that has been consistently present in infected cultures of newborn as well as of adult glands is not so readily apparent in figures 7 and 8, but was quite evident in the sections. This consisted in the virtually complete necrosis of the connective-tissue elements, including macrophages, within the explants that showed the most widespread proliferative response. Van Gieson connective-tissue stains showed less collagen in the infected cultures than in the controls, and the collagen in the infected cultures was distributed mainly about portions of ducts, where it probably represented the persistence of collagen that was already present in the original explants. Surviving fibroblasts were rarely seen within this collagen. Thus within a single organ, mesenchymal elements responded solely by necrosis, while the epithelial elements responded with both necrosis and proliferation.

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[4] Purchased from Microbiological Associates, Inc., Bethesda, Md.

[5] The author is grateful to Dr. Wallace P. Rowe of the National Institute of Allergy and Infectious Diseases for HI determinations on serums of these mice.

The response of submandibular gland from the 8-month-old female with an HI titer of 1 : 800 differed significantly from that of the glands of younger adult males with negative HI reactions. Which of the age, sex, or serologic variables was responsible for this response difference cannot be stated. The differences were: (1) a greater tendency for the proliferating epithelial elements to "invade" far out into the gelatin sponge, centrifugally from the explants (figs. 14 and 15); and (2) a greater tendency for the outgrowth to display acinar structures similar to patterns ordinarily displayed by adenocarcinomas (figs. 15, 16, and 17). These acinar structures were present in the absence of any stromal elements and at considerable distances from the explants. They resembled the glandular patterns reproduced by adenocarcinomas cultivated on gelatin sponge by Leighton in some of his original application of matrix methods to tissue culture (*78*).

Attention was next focused on the response of submandibular glands in early stages of development in embryos. Use was made of the extensive groundwork by Borghese (*79*, *80*) and Grobstein (*81*, *82*) in the culture of submandibular rudiments. Intact submandibular rudiments from C3H/Bi or (AKR × C3H/Bi)F_1 embryos were used at the 13th and 14th day of development; the day of detection of vaginal plug in the mother was counted as day 0. The gross and histologic appearances of these rudiments are shown in figures 18 to 21. Variation in degree of development was noted among the embryos, but, in general, on the 13th day the epithelial portion consisted of a single pear-shaped bud, and on the 14th day it had developed 4 to 15 branches with terminal swellings. The details of development of this organ in culture will not be reviewed here, as they have been accurately documented, and numbered stages have been assigned by Borghese (*79*, *80*) and by Grobstein (*81*, *82*). It is worth noting, however, that in sections of either 13- or 14-day rudiments, the cytologic features of the epithelial and capsular mesenchymal components are remarkably similar. In 14-day rudiments, in particular, proliferation of the epithelium is extremely rapid, as judged by frequency of mitotic figures. If polyoma virus could be assumed to exert its action immediately in rudiments exposed at this time, this action would have to represent a suppression of proliferation rate, as estimated by relative frequencies of mitotic figures in the rudiments and in the tumors *in vivo.*

Cultures of the intact rudiments were prepared in two ways as indicated in table 3. Those listed as "on or under plasma clot" were prepared by placing the rudiments on the surface of preformed chicken-plasma clot (3 parts chicken plasma and 4 parts chick-embryo extract) in Leighton tubes, or by placing the clotting mixture into the tubes after placement of the rudiments. Those listed as "on plasma clot adjacent to gelatin sponge" were prepared by placing the rudiments on the surface of chicken-plasma clot, adjacent to sponges that had previously been embedded in the clot.

TABLE 3.—Submandibular rudiment cultures studied for response to polyoma virus

Type of culture	Duration of culture period (*days*)	Age and number of rudiments cultured			
		13-Day rudiments		14-Day rudiments	
		Control	Test	Control	Test
On or under plasma clot	9	1	2	0	0
	12	0	0	2	2
	15	4	0	0	0
	17	0	0	6	6
	18	0	0	2	2
	22	0	0	2	2
Total on clot alone		5	2	12	12
On plasma clot adjacent to gelatin sponge	7	0	0	3	3
	8	3	3	0	0
	12	0	0	7	7
	14	2	2	4	3
	17	10	0 (Contaminated)	0	0
	19	2	2	0	0
	22	2	2	0	0
	27	0	0	7	7
	29	0	0	3	4
	30	6	7	0	0
Total on clot plus gelatin sponge		25	16	24	24
Total on both types of culture		30	18	36	36

Rudiments were exposed to virus from the same virus pool, and in the same manner employed with adult-gland fragments in this report. Control rudiments were allowed to stand in medium to which no virus was added, for the same 2-hour period, and all were kept at room temperature in a desiccator jar gassed with 5 percent CO_2 and 95 percent air during the exposure period.

Liquid medium, of the composition described previously, was first added to the cultures 16 hours after the rudiments were placed. Both control and test cultures were fed 2 to 3 times weekly, and *p*H was controlled by gassing the tubes with 5 percent CO_2 in 95 percent air.

The results are tabulated in table 2 along with previous data. The 14-day rudiments developed much as described by Borghese (*79, 80*) and

Grobstein (*81, 82*), regardless of whether they had been infected or not. Likewise, no difference between control and test cultures of 13-day rudiments could be detected in living cultures during the 1st week, though the degree and speed of morphogenesis displayed by these earlier rudiments was less extensive and more variable than for the later ones.

Viral effects first became visible in living cultures after 9 to 14 days. During this interval, necrosis of the mesenchymal cells became obvious, since the cells became dark and granular and eventually lost form. Figures 22 and 23 compare control and test cultures of 13-day rudiments after 17 days in culture under plasma clot, without gelatin sponge. Besides the fibroblastic cells of the mesenchyme, macrophages also underwent necrosis. These, too, became dark and rounded as the cytoplasm filled with brown granules. In the control cultures, swarms of clear, vigorous-appearing macrophages appeared in the rudiments and on the glass-clot interface after the 1st week of culture, and these persisted in good condition as along as the cultures were maintained. In living cultures on or under plasma clot there was no sign of epithelial damage or atypical proliferation at any time, but after the necrosis of the mesenchyme became marked, the epithelial elements were obscured by the granular debris (fig. 23). In cultures using gelatin sponges, the uninfected as well as the infected rudiments were drawn against and into the sponge as clot was lysed laterally. It was therefore more difficult to visualize differences in these until microscopic sections were prepared. However, the relative paucity of macrophages on the glass and in the clot adjacent to the infected rudiments contrasted with the presence of large numbers of healthy-appearing macrophages in comparable locations in control cultures.

In fixed and stained cultures the earliest histologic change was the presence of small foci of necrosis in the capsular mesenchyme, associated with nuclear changes in the mesenchymal cells, similar to the changes previously described in epithelium (*39*). These changes, without involvement of epithelium and without proliferative features, were seen in 13-day infected rudiments after 9 days in culture (fig. 25). Control cultures showed no similar changes (fig. 24).

Cytolysis and nuclear lesions of the epithelial portions of the rudiments were first seen in 12-day-old cultures of infected 14-day rudiments (figs. 26 and 27). At this time there were also necrosis and nuclear inclusions in the mesenchymal cells, but still no extensive loss of these elements.

After 22 days in culture, infected 13- or 14-day rudiments showed a more extensive necrosis of mesenchyme, as well as epithelial necrosis, and nuclear lesions of both epithelium and mesenchyme (figs. 28 to 31). Although the pattern of the epithelial tree was disrupted by the effects of necrosis, there was still no evidence of a proliferative reaction.

The earliest evidence of a proliferative response that has been observed as yet in infected rudiment cultures occurred in 14-day rudiments after 27 days in culture. Figures 33 to 35 illustrate the morphologic alteration that was seen in comparison with a control culture (fig. 32). The alteration was similar qualitatively to that seen in infected cultures of glands of newborns, but was considerably less voluminous in the rudiments. It is difficult to assess this apparent quantitative difference because of the great differences in the nature of the starting materials. Certainly as yet there has occurred no distant extension of the altered epithelium into the gelatin sponges, as was seen with infected cultures of adult submandibular glands.

To summarize these still incomplete observations on the response of salivary gland to polyoma virus in this tissue-culture system, it can be said that there was a trend from the youngest to the oldest tissue toward an increasingly active proliferative response, whereas there was a trend in the opposite direction with respect to the cytolytic response. The difference between early rudiments and newborn tissue also appeared to be greater than the difference between newborn and adult tissue.

III. SPECULATION ON MECHANISMS

Does the preceding information as to *where* and *when* the polyoma virus elicits a proliferative response give us material from which to construct a working hypothesis as to *how* it acts? Our knowledge is still so fragmentary as hardly to permit the question, much less the answer. The following comments are therefore entirely speculative:

We have seen that the range of species susceptibility to the agent, so far as proliferative response is concerned, is limited to 4 or possibly 5 rodents, each of which responds with a different pattern of tissue response. The kidney and subcutaneous connective tissues are the only sites that may be involved in all. This rather narrow range of species specificity is similar to that seen in general for other tumor viruses, in contrast to the much wider range that is shown by many "nontumor" viruses, *e.g.*, influenza, rabies, and lymphocytic choriomeningitis (LCM) viruses.

The tissue-specificity range, though broad for the mouse, is nevertheless limited. With few exceptions, the tissues that most frequently develop tumors are examples of higher-degree differentiation far out on the ontogenetic tree, where versatility of response is limited, *e.g.*, salivary and lacrimal glands, thymus, thyroid gland, hair follicles, mammary glands, and dental epithelium. Possibly a connection exists between the facts that the species range is wider for kidney tumors, and the morphogenetic response of metanephrogenic mesenchyme also has a low heterospecificity to induction by ureteral bud or spinal cord (*73*, *74*). Is there also a clue, hidden in the hydrocephalic response (*66*) to polyoma virus, relating the inductive capacity of spinal cord to susceptibility of some as yet undetermined part of the central nervous system to polyoma virus? Not much is known of the interspecific responsiveness of the epithelial rudiment of the submandibular gland, but it has been shown that the morphogenetic response of this epithelium is highly specific for capsular mesenchyme of the same organ (*82*).

If one can judge from tissue-culture observations, the proliferative type of response in the submandibular gland, at least, is correlated with the degree to which the epithelium of this organ has acquired its tissue-characteristic properties. When infected in the earliest stages of morphogenesis, this tissue responds predominantly by necrosis. A parallel may exist between these relationships in the mouse-polyoma virus system and the hemorrhagic versus proliferative response of the chicken-Rous virus system (*83*).

Since morphogenesis of the salivary-gland epithelium of the mouse is strictly dependent on homologous mesenchyme (*82*), and proliferative response in turn is correlated with the developmental stage of this epithelium, it would appear that the tumor-inducing capacity of polyoma virus is indirectly dependent on the establishment and perhaps the maintenance for some time of the specific interrelationship between the mesenchymal and epithelial components. In connection with this point it is pertinent that in tissue-culture systems using disaggregated embryonic mouse cells, no proliferative phenomenon has been reported, whereas in organ cultures it does occur, along with the cytolytic phenomenon. A disturbance of a specific mesenchymal-epithelial relationship could also account for the irregularity of results in transplantation of many of the polyoma-induced tumors (*13*, *45*, *84*).

The superiority of the newborn period for exhibition of proliferative response to polyoma virus would seem to be attributable to the ideal overlap of two critical biological phases in chronologic time. Thus, defectiveness of immune response allows infection and "boosting" of virus concentration at a period when the susceptible tissues have reached a potential for proliferative response. A *continued* proliferative response might not be possible, however, were it not for timely intervention of the immune reaction, which prevents complete dissolution of the intercellular dependency postulated for the proliferative response. Too late an immune response would lead to death of the animal, or the runting phenomenon, the latter as a result of extensive loss of mesenchymal tissues, *e.g.*, bone.

To look at the composite picture from this point of view, the salivary-gland tumors early in their development would be *doubly dependent:* (1) on establishment within the epithelial cells by virus, and (2) on the existence of the proper relationship between mesenchymal and epithelial components of the organ. In this tripartite relationship, the mesenchyme might act temporarily as the "balancing influence" which has been emphasized (*85*) as necessary to prevent virus from "gaining the upper hand" and to produce cytolysis of the host cell. Thus there would exist within the epithelial cell a competition between virus and cell for a complex material supplied by the mesenchyme and presumably identical with factors normally controlling morphogenesis and/or differentiation. The morphogenetic factor supplied by the mesenchymal cell could be analogous to the specific repressor substance responsible for maintenance of phage in a prophage state in bacterial systems (*86*). In specialized multicellular organisms, it would be logical to expect that the ability to produce or induce the production of repressor substance should reside in cells other than those carrying the virus, or normally responding to the repressor substance in morphogenetic or differentiative phenomena. Upon separation of epithelium from specific mesenchyme, the virus would multiply and/or become complete, and destroy the host cell. Some other virus-tumor systems might also fit this concept. For example, it is in accord with the observation that Shope papilloma virus is abundantly present in the dying cells of the outer layers of the epithelium, whereas it is either not present in complete form or is present in undetectable quantities in the basal, proliferating cell layers (*87*).

The recent report of Hearn (*88*) is relevant to this discussion. He found that, in tissue cultures chronically carrying Venezuelan equine encephalomyelitis, *removal* of glutamine resulted in the prompt appearance of a cytopathic effect, and simultaneously with this a great *increase* of virus production. In this system the virus-suppressing role of a second cell type would have to be filled by a relatively simple nutrient, glutamine, or more likely by a complex material produced by the infected cell itself, utilizing glutamine. An analogy was drawn by Hearn between this system and lysogenic phage systems.

To summarize: Differences in sensitivity and pattern of response to polyoma virus by different species, strains, tissues, and different cells within these tissues at various stages of biologic development have been reviewed in an attempt to see some order in a picture that at first appears chaotic.

Development of progressively growing tumors in the intact animal is limited, in present knowledge, to 4 rodents: mouse, rat, hamster, and Mastomys. Each of these species has a distinct pattern of tumor response, with Mastomys developing some, but not all, tumors characteristic of both hamster and rat. The apparent gap between the mouse and the other 3 rodents with respect to failure of the latter to respond with epithelial tumors has been partly bridged by Rabson's observation of epithelial lung tumors in hamsters receiving large doses of virus intratracheally. A common theme in the response of these 4 animals is the occurrence of connective-tissue tumors of the kidney and subcutaneous tissues in all. Whether the tumors at these sites arise from the same cell type in each animal is unknown. As with other viruses, it appears that species barriers, at least concerning tissue-response differences, may be partially broken down by dosage and route-of-administration factors.

Some evidence for partial resistance of strain C57BL/Ka mice to the tumor-inducing effects of polyoma virus is cited. It is not known whether this presumed genetic influence is exerted directly at the cell-response level or indirectly at the immune-response level.

Organ-culture studies of the response to polyoma virus by submandibular gland of the mouse at different stages of morphologic and biologic development were reported. It was found that the proliferative response was most marked in adult glands and least marked and latest in appearing in 13- and 14-day rudiments of glands from embryos. In embryonic rudiments, cytolytic effects were noted first in the mesenchymal cells, then in epithelium. The morphologic character of the proliferative phase of the response differed for submandibular gland taken from an adult, immune female as compared with response of glands from newborn or embryo mice. In the former, there was wider spread of proliferating epithelium into the gelatin sponge, and a greater tendency toward a gland-forming pattern.

Our present impression, based on these still incomplete organ-culture studies, is that adult submandibular gland is more susceptible to the proliferation-inducing effects of polyoma virus than is the same tissue from newborn or embryonic mice. The advantage of newborn animals for tumor induction *in vivo* appears to depend on the overlap in time of (1) a period of weak immune response and (2) a period of intermediate capability for proliferative response.

A concept concerning intercellular relationships influencing the development of tumors in the salivary-gland/polyoma virus system is outlined. In this double-dependency scheme, a balance between virus and epithelial cell, favoring cell proliferation but restraining viral proliferation and resultant cytolysis, is maintained during the early stages of tumor genesis by the mesenchymal cells specifically responsible normally for morphologic and functional control. Investigations for testing this concept are now possible by techniques developed in the field of experimental embryology.

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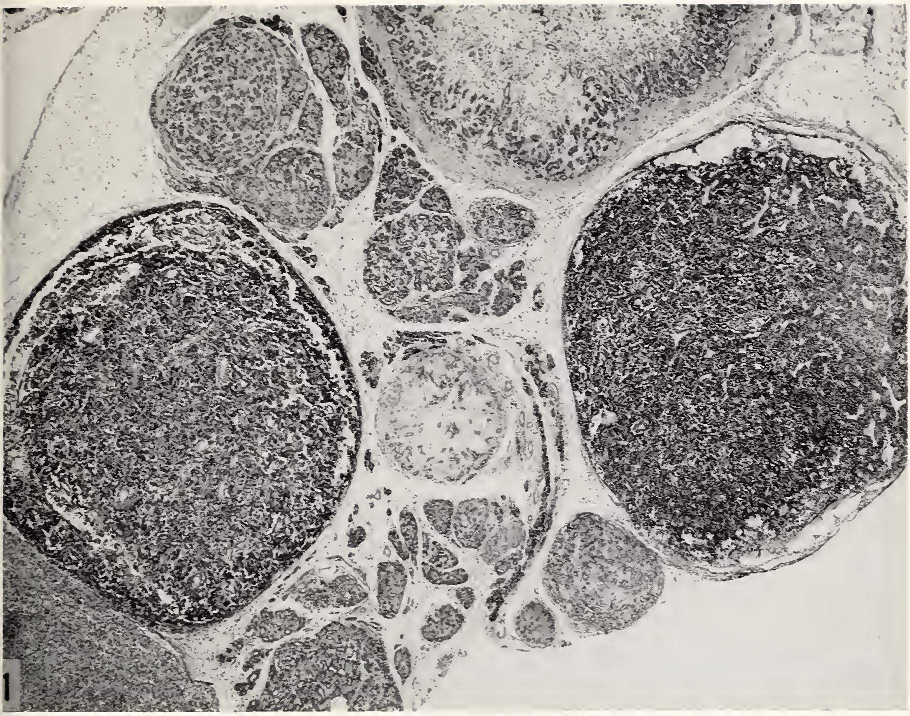
PLATES

PLATE 7

FIGURE 1.—Low magnification showing 2 parotid-tumor nodules (*dark* areas at *left* and *right*) in which lymphoid cells make up majority of cell population. *Note* general resemblance of nodules to lymph nodes. Other tumor foci not exhibiting lymphocytic accumulations are also present. From a strain C3H/BiLw mouse inoculated within 24 hours after birth with polyoma virus, and autopsied when 3.5 months old. Hematoxylin and eosin. × 15

FIGURE 2.—Higher magnification of a parotid tumor of same type shown in figure 1. Tubular epithelial structures made up of cells with large, pale nuclei at *right*. *Note* varying stages of maturation of lymphoid cells, as in normal lymphocytogenesis. From a strain C3H/BiLw mouse inoculated within 24 hours after birth with polyoma virus, and autopsied when 3 months old. Hematoxylin and eosin. × 610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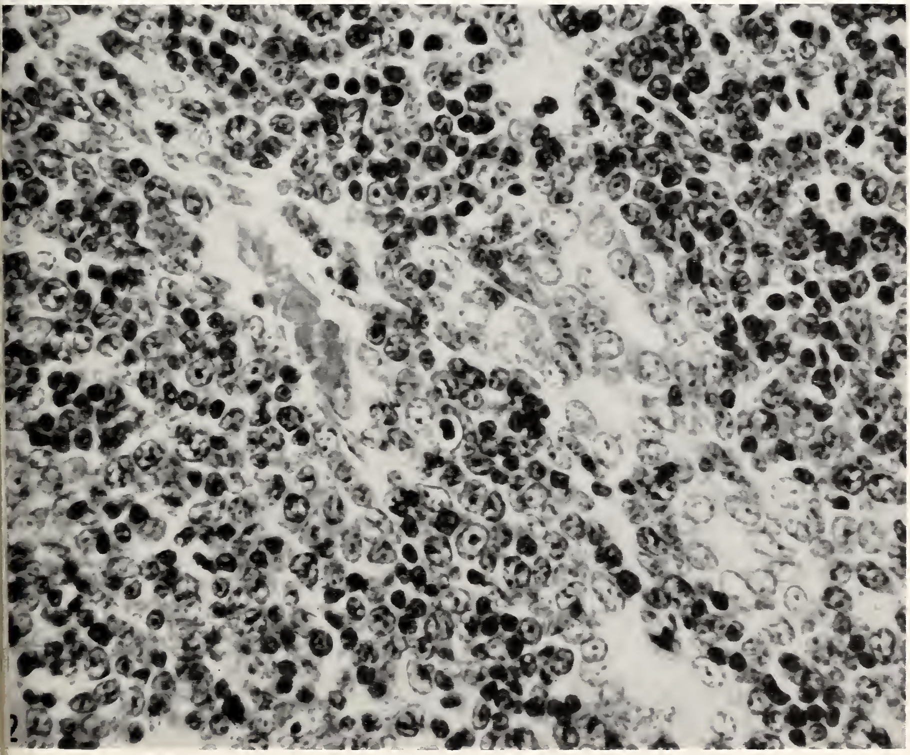
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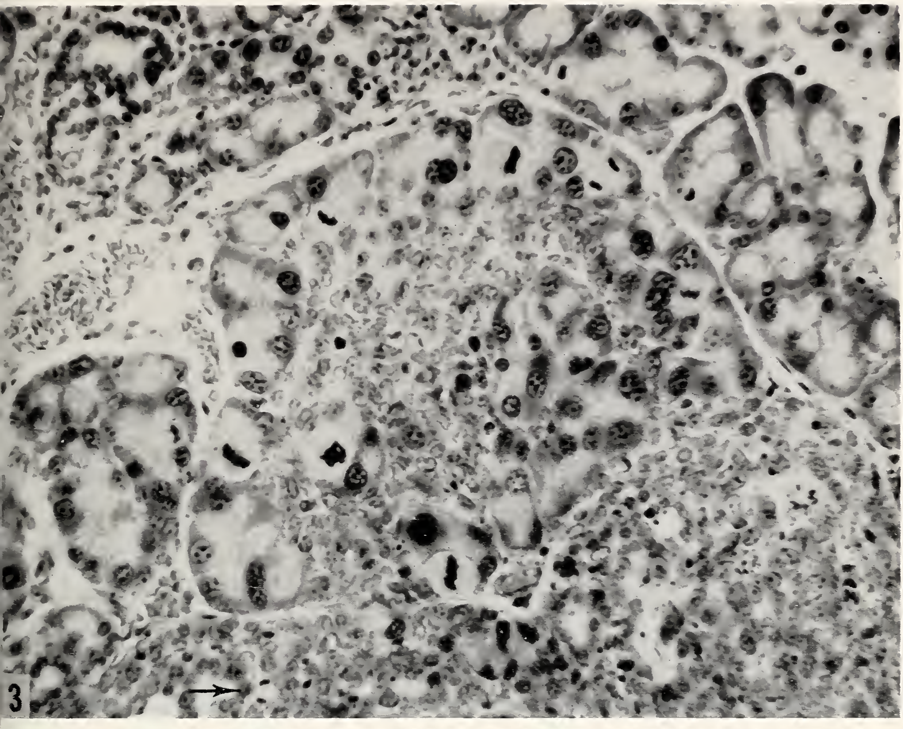
PLATE 8

FIGURE 3.—Lobule of extraorbital lacrimal gland partially replaced by polyoma virus-induced tumor (*below*, and *center*). *Note* large size of nuclei of non-tumor acinar cells in periphery of circumscribed lobule. Many giant mitotic figures are present among cells. *Arrow* (*lower left*) indicates mitotic figure in tumor cell. Hematoxylin and eosin. × 290

FIGURE 4.—Normal extraorbital lacrimal gland of young adult mouse. *Compare* nuclei of acinar cells with nuclei of similar cells adjacent to tumor focus in figure 3. Hematoxylin and eosin. × 290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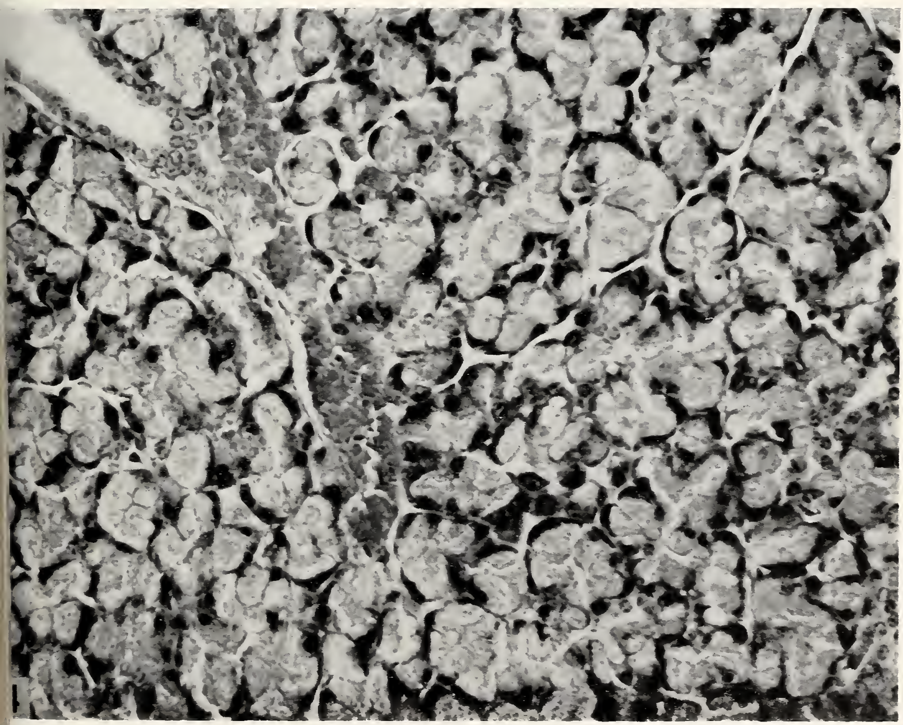
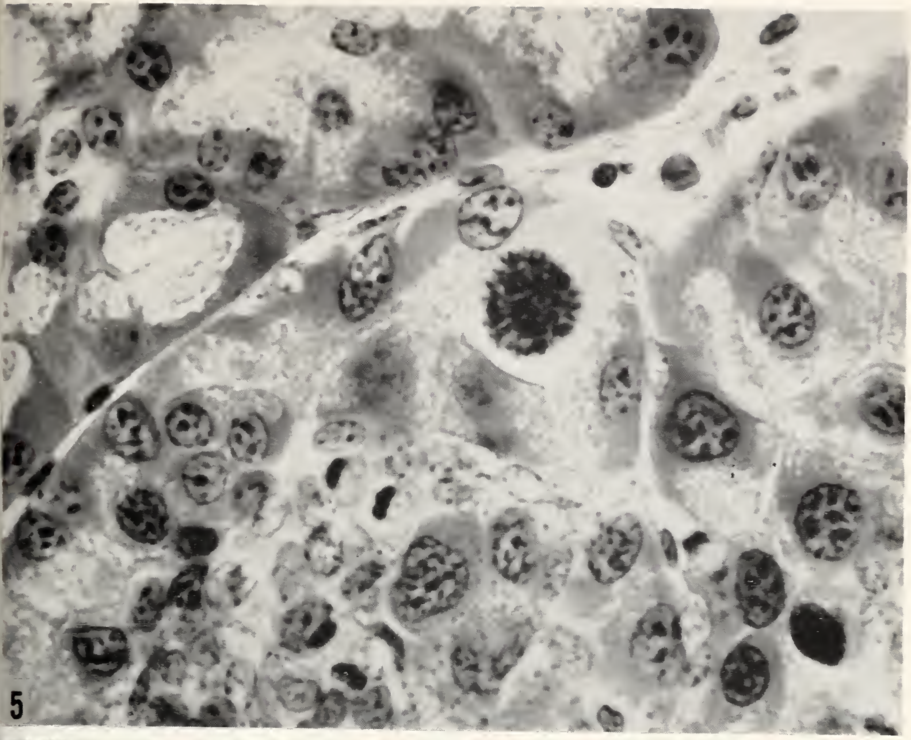


PLATE 9

FIGURE 5.—Giant mitotic figure (*right center*) in acinar cell of extraorbital lacrimal gland, adjacent to tumor focus (*lower left*). More than 100 chromosomes were estimated present in large mitotic figure. Telophase mitotic figure within polyoma virus-induced tumor is present *below* and to *left*. Hematoxylin and eosin. × 880

FIGURE 6.—Gelatin sponge matrix culture of submandibular salivary gland from a 5-month-old male C3H/Bi mouse, 30 days after explantation and exposure to polyoma virus. Solid clumps and cords of altered cells surround original explant (*center*) and extend into adjacent gelatin sponge. Hematoxylin and eosin. × 200



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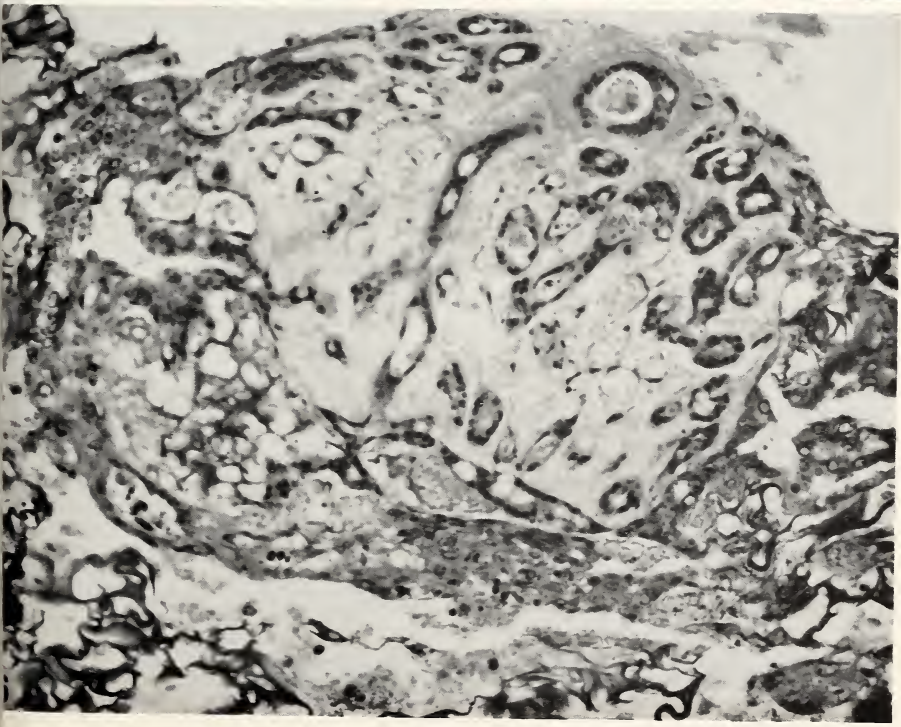
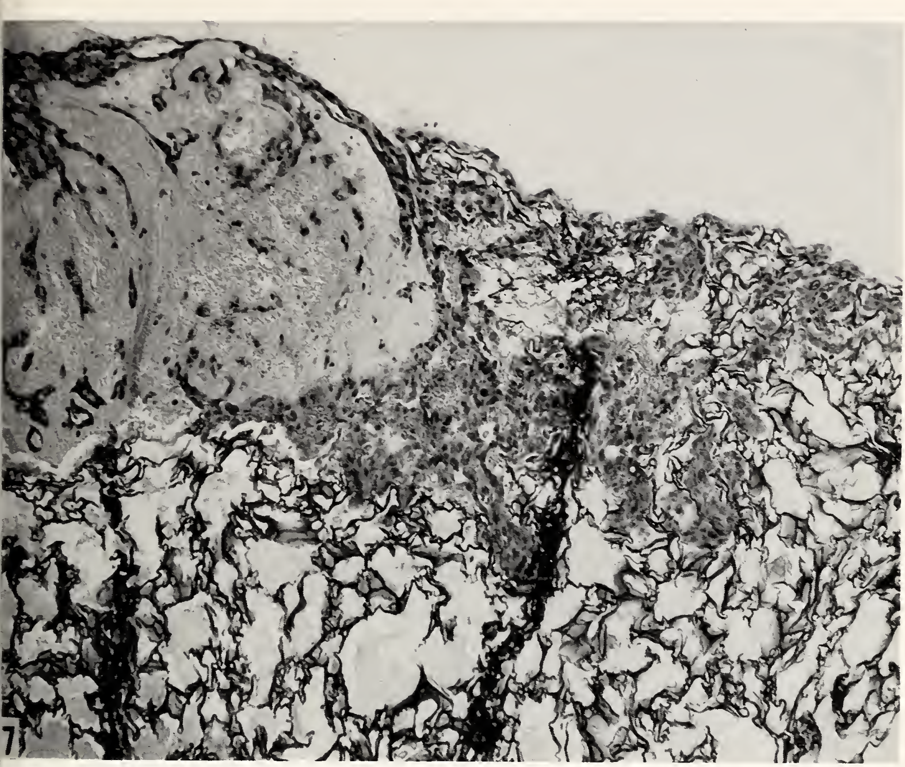


PLATE 10

FIGURE 7.—Gelatin sponge matrix culture of submandibular gland from 8-week-old C3H/Bi male mouse, 30 days after explantation and exposure to polyoma virus. *Note* extensive growth of altered epithelium into the sponge to *right* of explant. *Compare* with uninfected control culture in figure 8. Hematoxylin and eosin. × 130

FIGURE 8.—Gelatin sponge matrix culture of submandibular gland from same animal donating explant shown in figure 7. Explant shown here was also cultured for 30 days, but was not exposed to polyoma virus. *Note* absence of epithelial proliferation from margins of, or within explant. *Also note* many nuclei of viable stromal cells, as compared with their nearly complete absence in explant in figure 7. Hematoxylin and eosin. × 130



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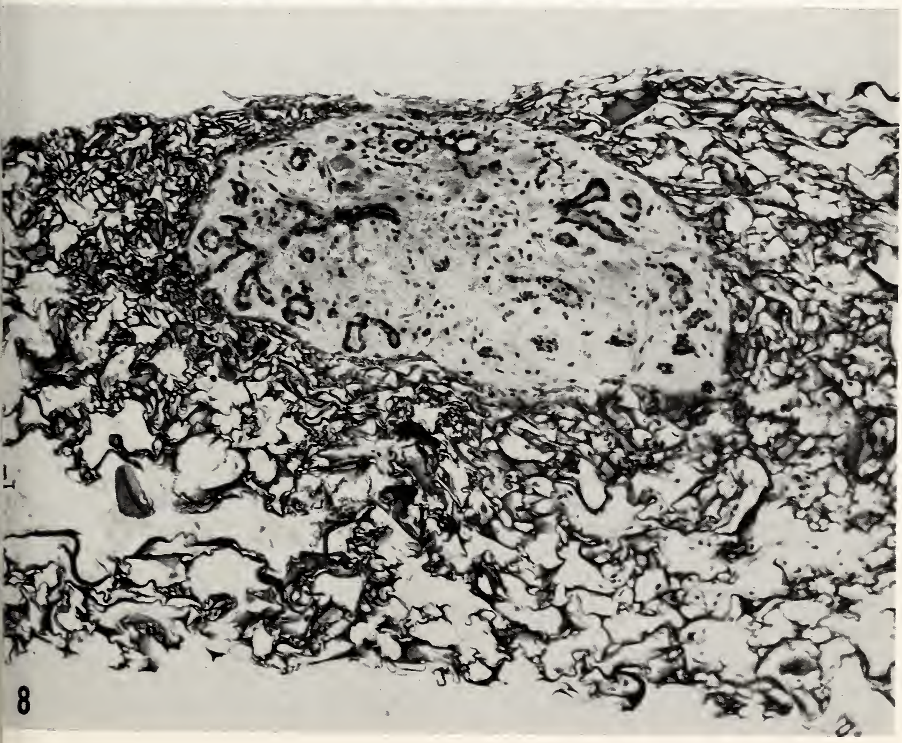

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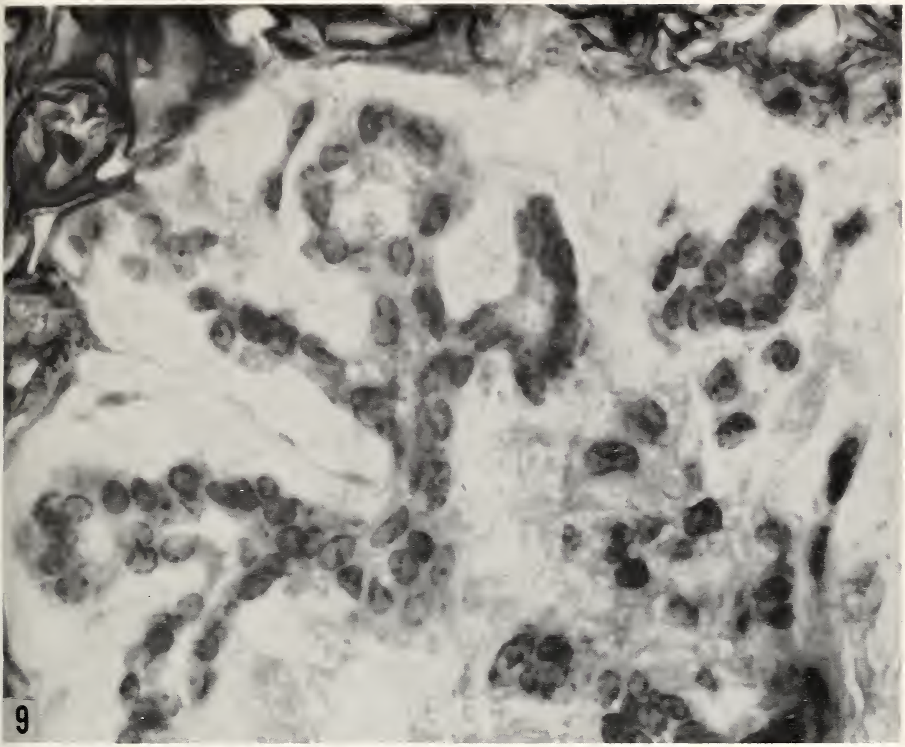
PLATE 11

FIGURE 9.—Higher magnification of area shown in figure 8. *Note* persistence of normal branched glandular pattern and uniformity of cell nuclei. *Compare* with cellular changes in figure 10. Hematoxylin and eosin. × 780

FIGURE 10.—Higher magnification of area shown in figure 7. *Note* large nuclei and nucleoli, and basophilic cytoplasm of altered epithelium growing within compartments of gelatin sponge. At *right center* is mitotic figure close to nuclear remains of disintegrated cells. Hematoxylin and eosin. × 780



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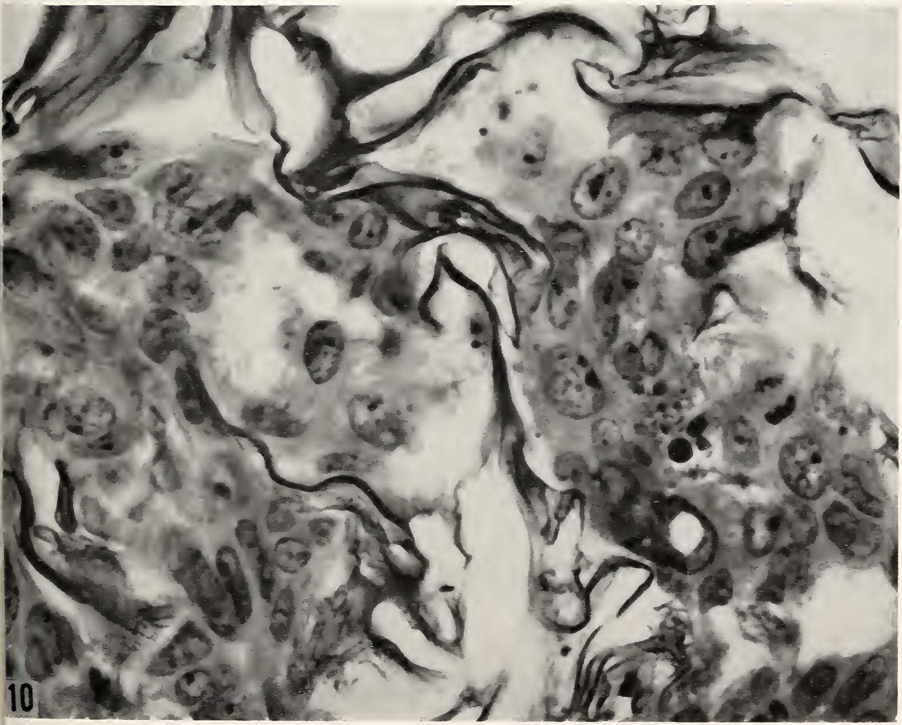

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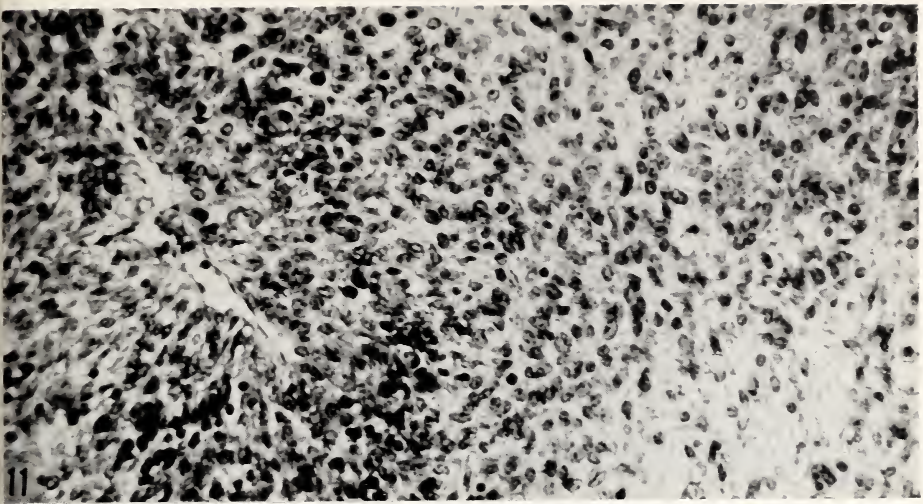
PLATE 12

FIGURE 11.—Section of tumor of salivary gland in a C3H/Bi mouse, nearly 12 months after mouse received subcutaneous inoculation of polyoma virus from tenth serial passage in P388 D_1 cell cultures. Hematoxylin and eosin. × 290

FIGURE 12.—Section of tumor that grew subcutaneously over lumbar spine, at site of transplantation of 30-day culture of adult C3H/Bi submandibular gland infected with polyoma virus from tenth serial passage in P388 D_1 cell cultures. *Note* marked similarity of cellular pattern here to that of tumor induced *in vivo*, shown in figure 11. Hematoxylin and eosin. × 290

FIGURE 13.—Section of polyoma virus-induced tumor of salivary gland, in its first transplant generation. Perivascular arrangement of cells is less pronounced, but general features are similar to those in tumor in figures 11 and 12. Section was from a transplanted parotid tumor described by Law, Dunn, and Boyle (*45*) and was illustrated in their figure 24, plate 39. It was induced by direct extract from leukemic tissue. Hematoxylin and eosin. × 290



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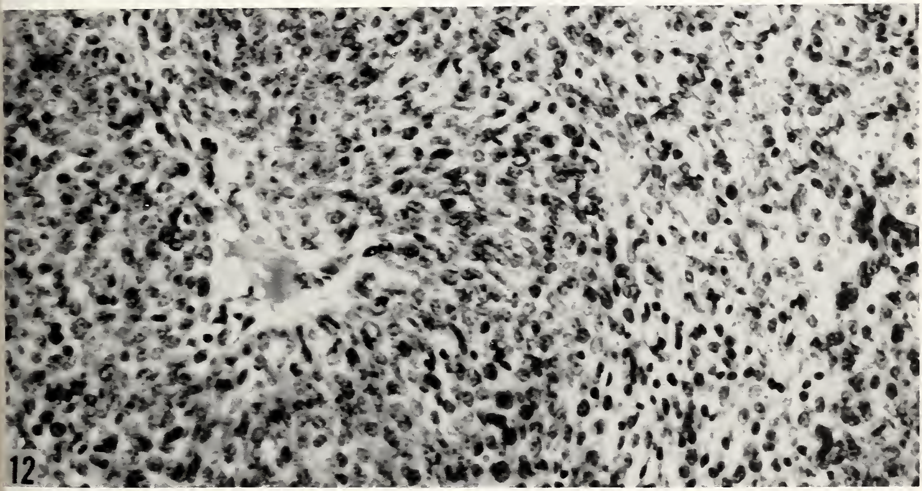

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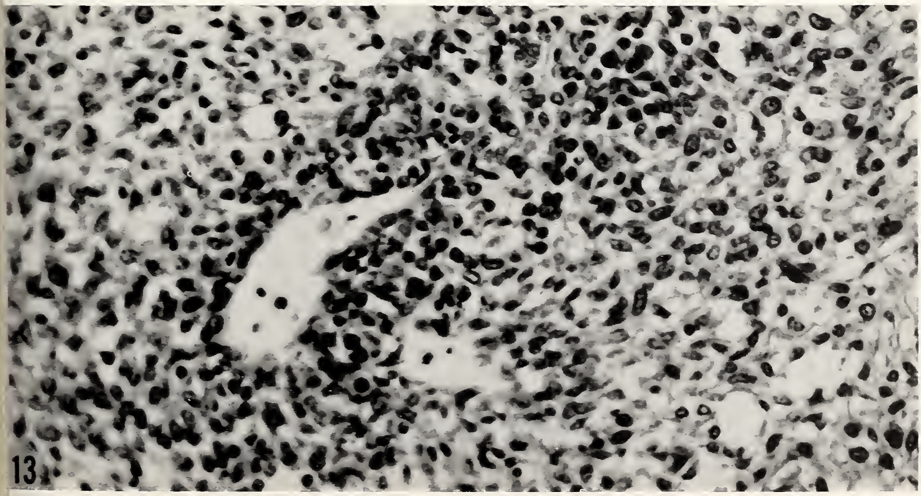

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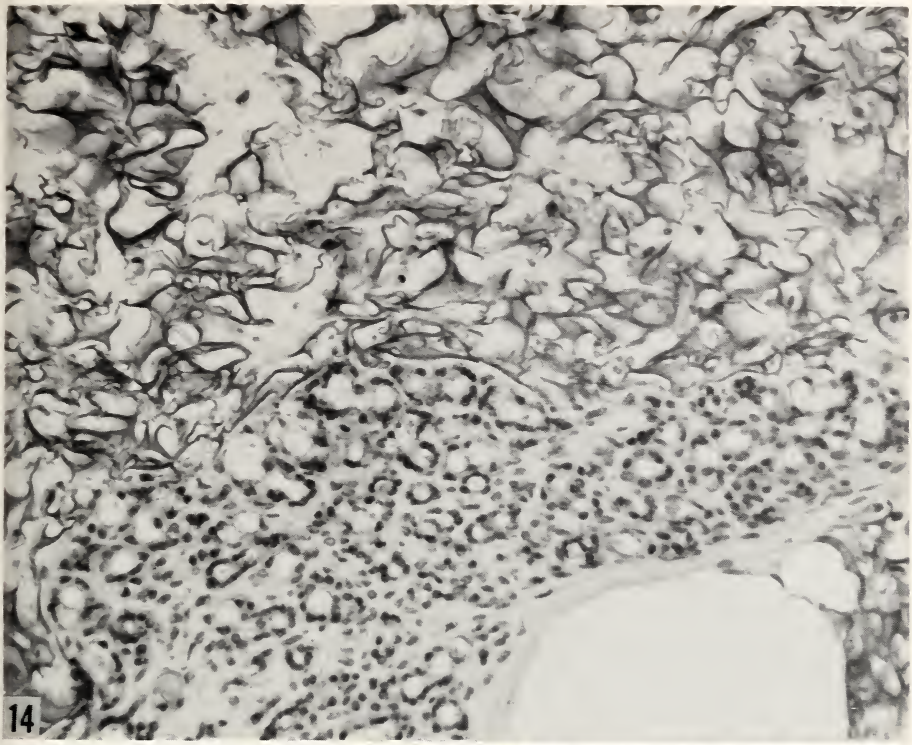
PLATE 13

FIGURE 14.—Gelatin sponge matrix culture of submandibular gland from 8-month-old C3H/Bi female, with HI titer of 1:800. After 30 days in culture, unexposed to polyoma virus *in vitro*. *Note* preservation of glandular pattern and lack of growth or migration of epithelium into adjacent sponge. Hematoxylin and eosin. × 260

FIGURE 15.—Culture from same donor and of same duration as that shown in figure 14, but infected with polyoma virus at time of explantation. *Note* glandular pattern of epithelial growth extending widely into sponge. Actual extent of growth away from explant (*left*) was more than twice the distance shown here in all directions. *Compare* with figure 14. Hematoxylin and eosin. × 260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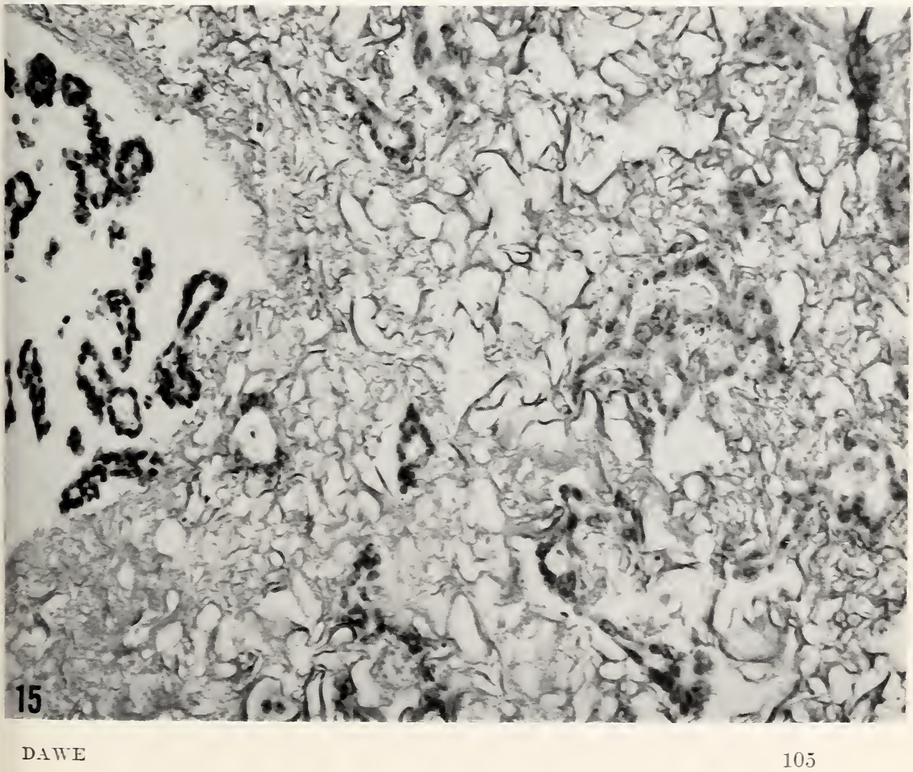
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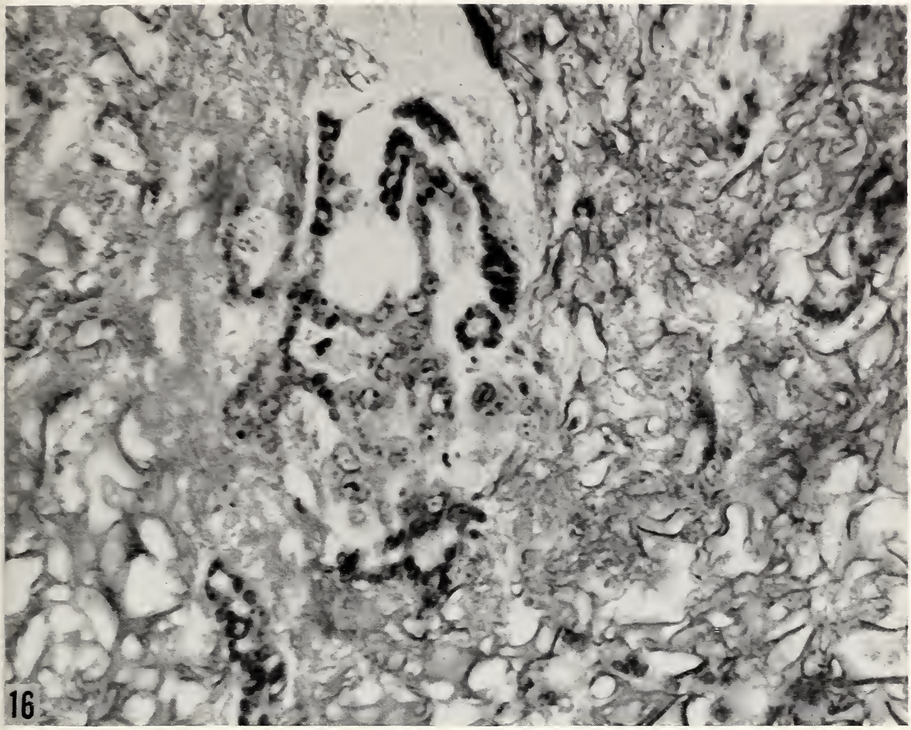
Plate 14

Figure 16.—Another area from same culture shown in figure 15 to demonstrate continuity of epithelium of original explant (*center*) with altered epithelium extending centrifugally into sponge. Hematoxylin and eosin. × 340

Figure 17.—Section of duplicate culture to that shown in figures 15 and 16. *Note* gland-like pattern of altered epithelium growing within compartments of sponge at distance from original explant (not shown). Mitotic figure at *upper left.* Dark basophilic body within lumen at *lower right* is pyknotic nucleus of dead cell. Hematoxylin and eosin. × 570



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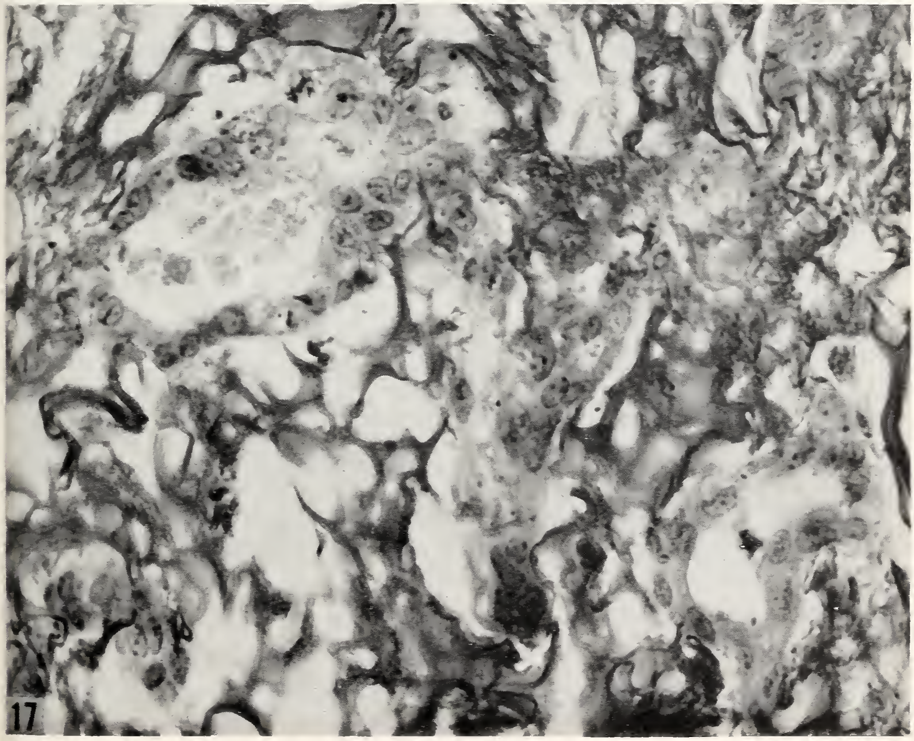

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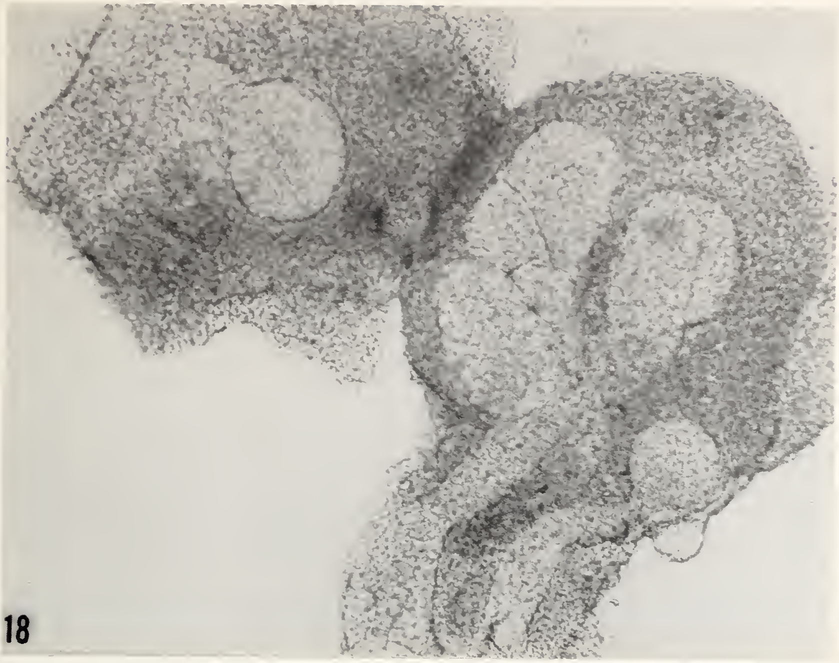
Plate 15

Figure 18.—Excised submandibular gland rudiments from 13-day (*above left*) and 14-day (*right*) C3H/Bi embryos. Epithelial portions of rudiments are light, sharply circumscribed structures surrounded by darker capsular mesenchyme. Sublingual gland rudiment is also present at *lower right* of 14-day submandibular rudiment. Unstained, fresh specimens. × 90

Figure 19.—Appearance of 14-day submandibular rudiment after 5 days in tissue culture. *Note* extensive development of adenomeres. Duct is mildly dilated. Unstained living culture. × 50



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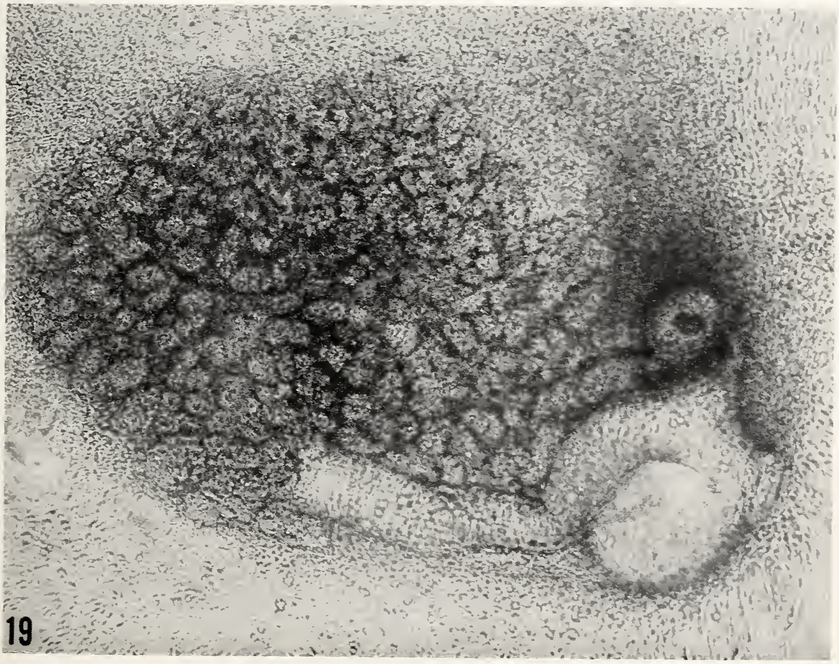

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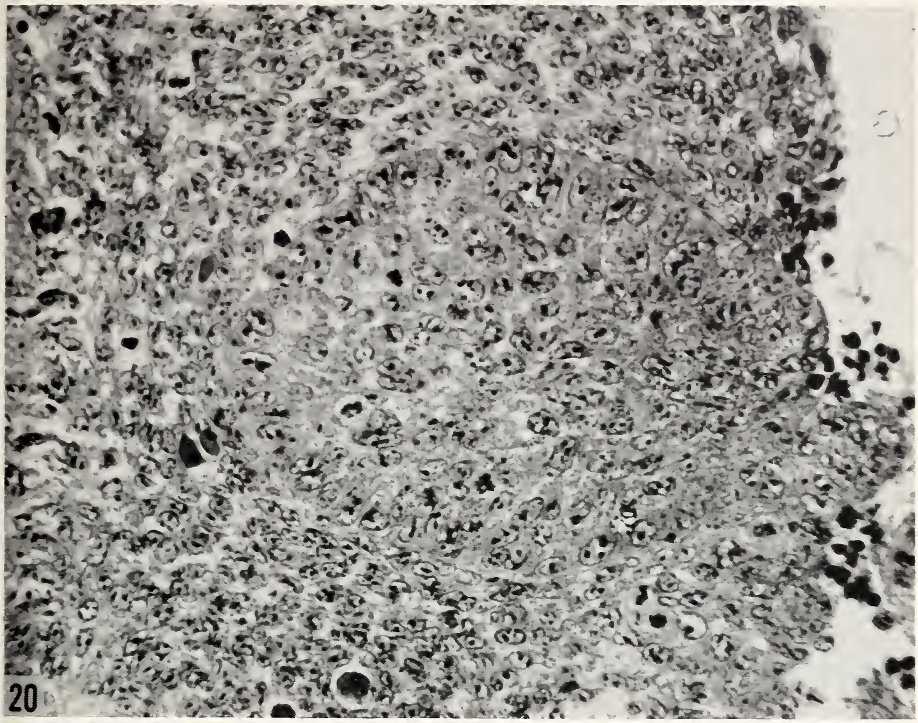
Plate 16

Figure 20.—Histologic appearance of submandibular gland rudiment freshly excised from 13-day-old C3H/Bi embryo. Stalk has been transected at *right*. Cells of epithelial portion show no differentiated features at this time. Hematoxylin and eosin. × 475

Figure 21.—Histologic appearance of submandibular gland rudiment freshly excised from a 14-day-old C3H/Bi embryo. Terminal swellings of a bifurcating branch of the epithelial rudiment are shown surrounded by capsular mesenchyme. Many mitoses present in the still undifferentiated epithelium. Hematoxylin and eosin. × 520



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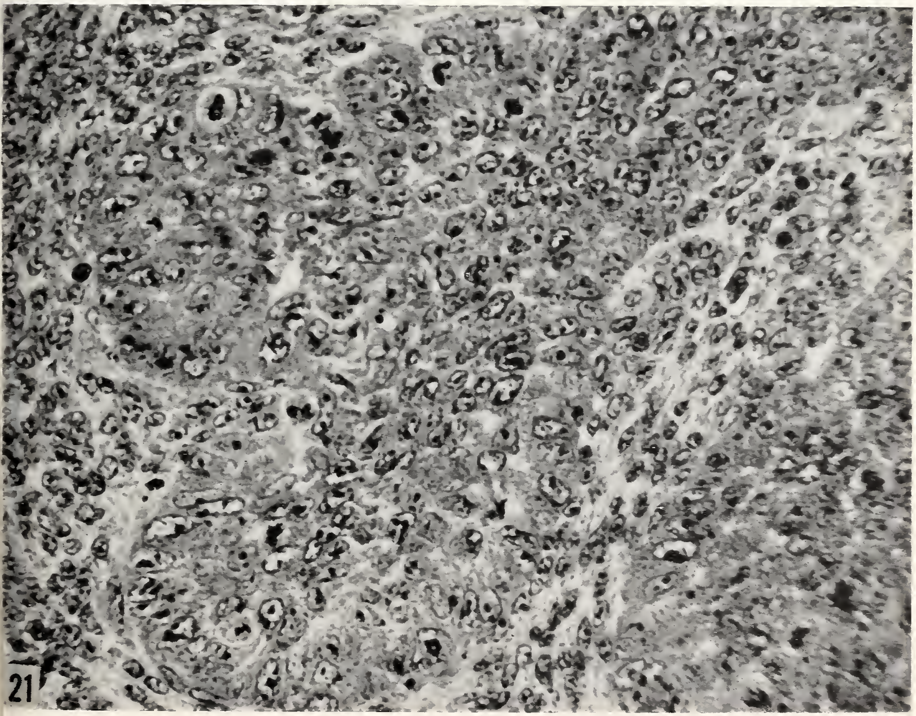

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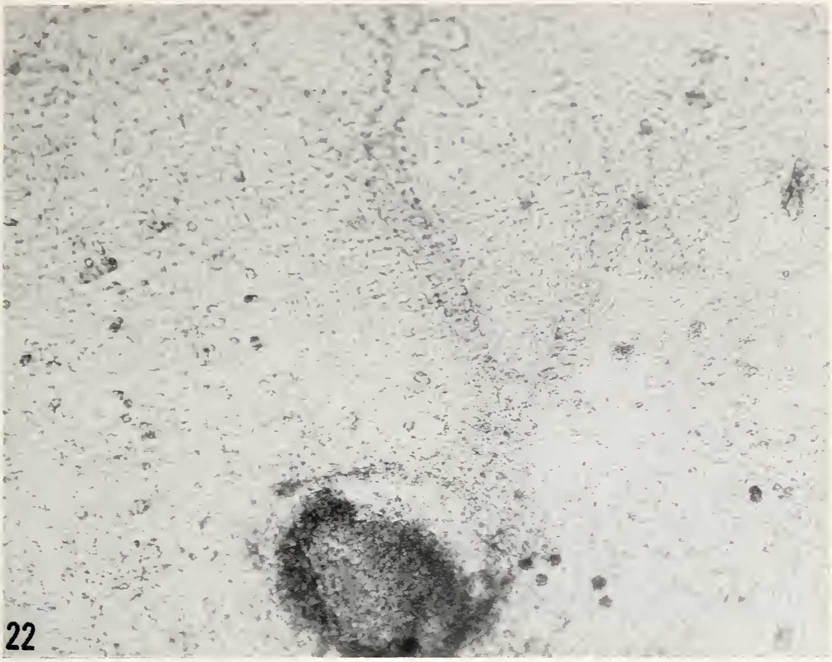
PLATE 17

FIGURE 22.—Submandibular rudiment from 13-day C3H/Bi embryo, after 17 days in culture. Not infected with polyoma virus. Development is limited in this example to formation of several branching tubular structures (*upper left* and *right*) extending out from dilated duct (*lower center*). Epithelium and mesenchyme are in good viable condition. Living culture. × 90

FIGURE 23.—Culture identical with that shown in figure 22, but infected with polyoma virus at time of isolation. Epithelial development was similar in degree to that in figure 22 before viral effects appeared. Epithelium obscured by extensive necrosis of mesenchyme. × 90



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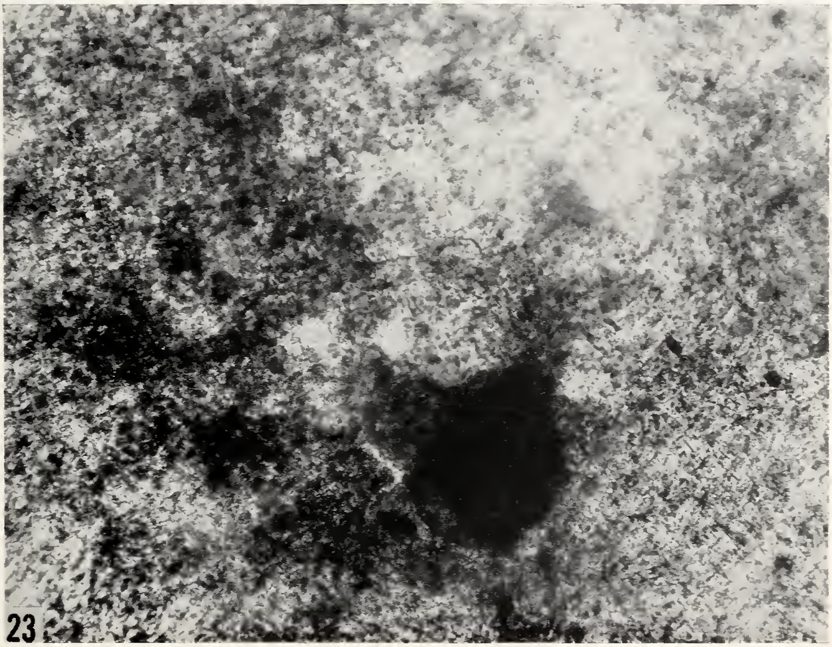

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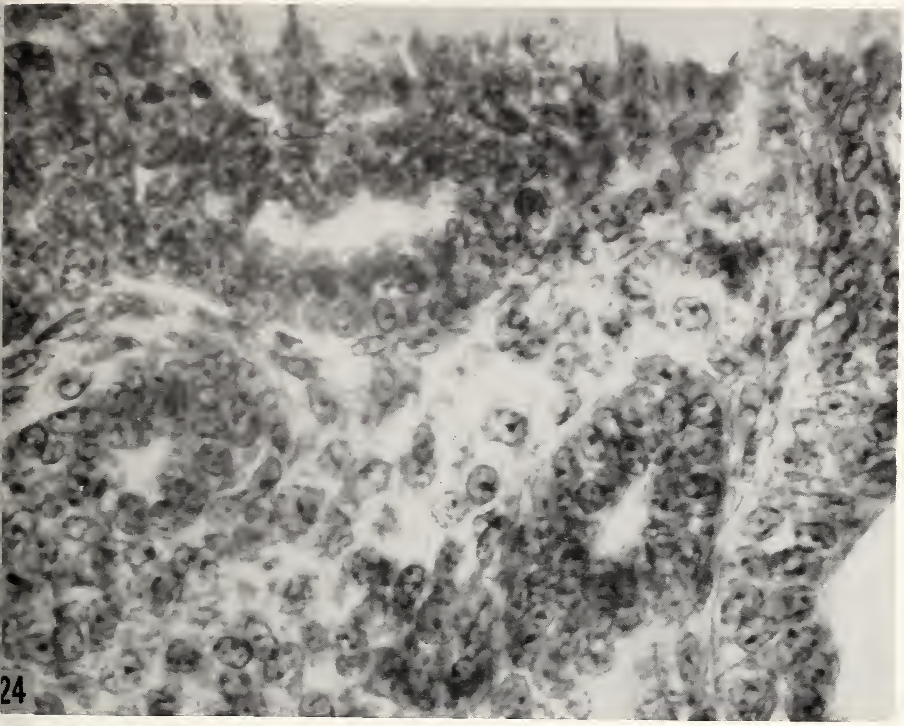
DAWE

PLATE 18

FIGURE 24.—Thick section of 13-day submandibular rudiment after 9 days in uninfected tissue culture. Tubular epithelial structures have branched from original bud. No necrosis of epithelium or mesenchyme. Hematoxylin and eosin. × 520

FIGURE 25.—Thick section of 13-day submandibular rudiment after 9 days in polyoma virus-infected culture. Slightly to *right* and *above center*, nucleus of mesenchymal cell contains inclusion body. Debris from lysed cell is adjacent to it, on *left*. Nuclei of other mesenchymal cells (*center*- and *lower-right*) show vacuolar changes. Hematoxylin and eosin. × 780



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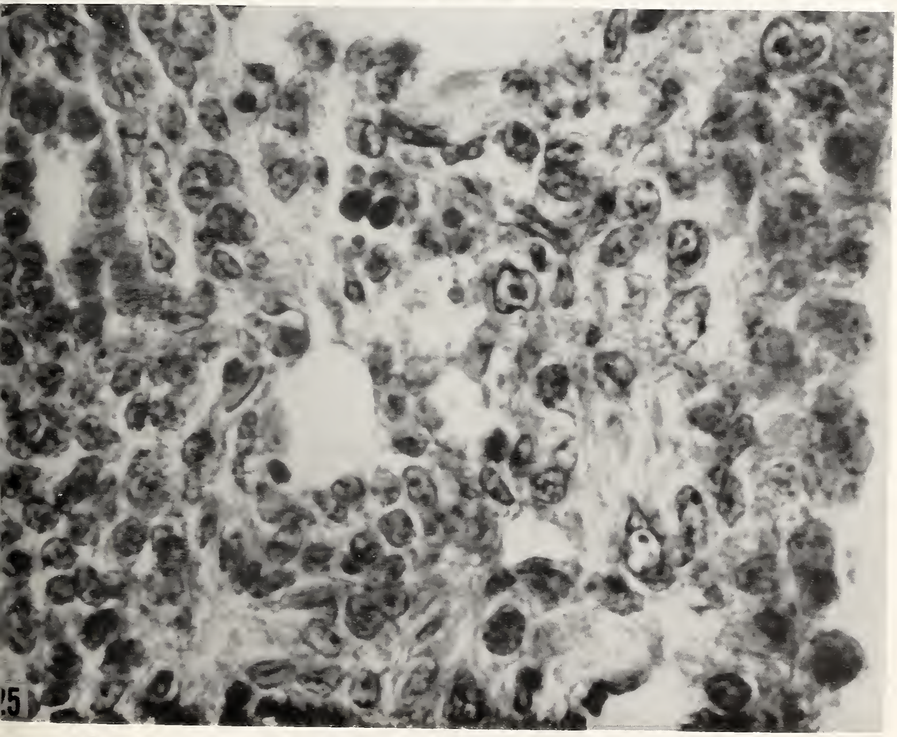

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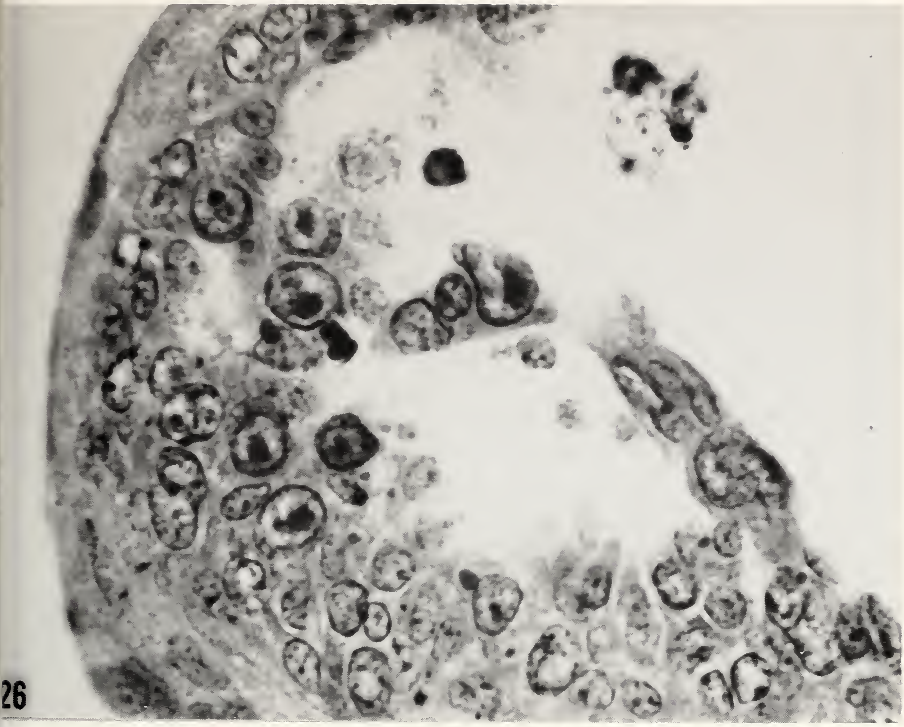
Plate 19

Figure 26.—Section of dilated ductal portion of 14-day submandibular rudiment after 12 days in polyoma virus-infected culture. Several epithelial cells show amphophilic intranuclear inclusions. Others show nuclear vacuolation (*below*). Hematoxylin and eosin. × 780

Figure 27.—Control culture of 14-day submandibular rudiment after 12 days. No epithelial or mesenchymal changes. Hematoxylin and eosin. × 315



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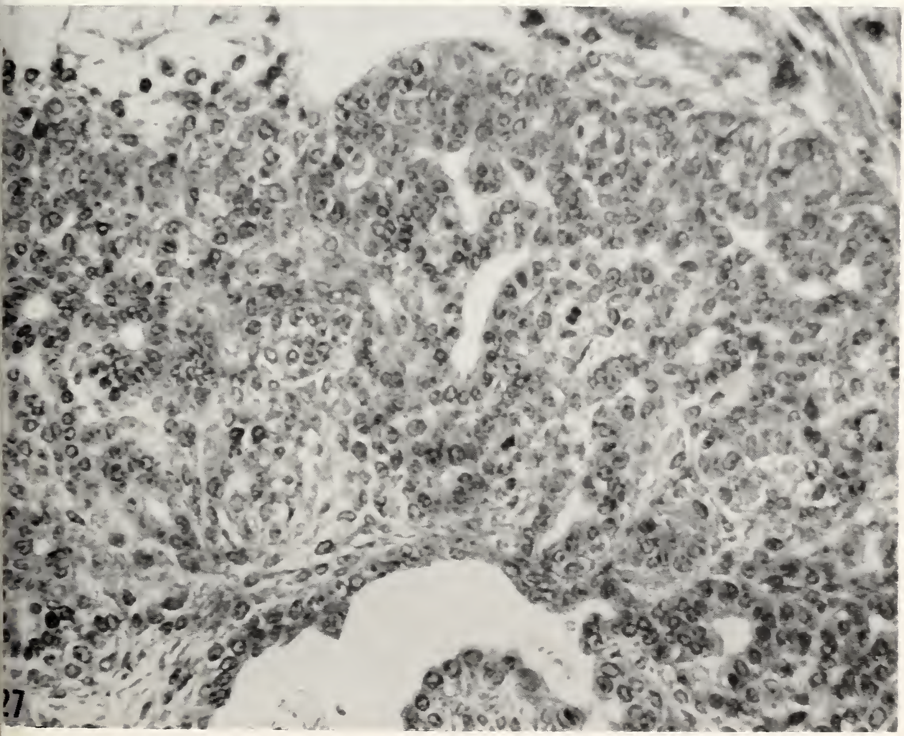

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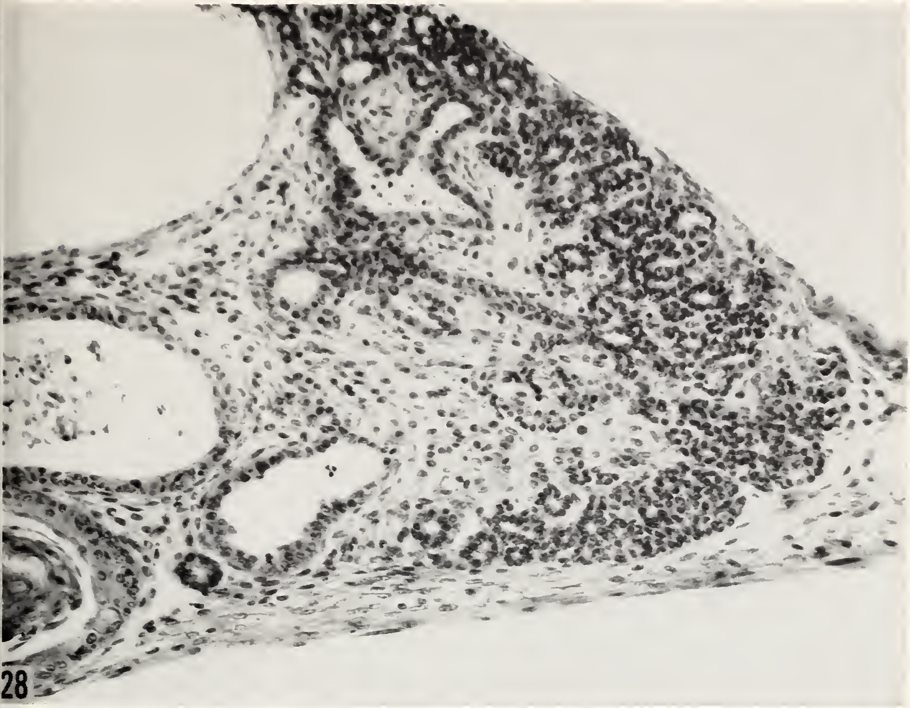
PLATE 20

FIGURE 28.—Section of 14-day submandibular rudiment after 22 days in culture, not infected with polyoma virus. *Note* well-advanced morphogenesis and good viability, although ducts (*left*) are dilated. Hematoxylin and eosin. × 200

FIGURE 29.—Section of culture similar to that shown in figure 25, but infected with polyoma virus at time of explantation. *Note* necrosis of mesenchyme and intranuclear lesions in epithelium at *upper right*. Hematoxylin and eosin. × 200



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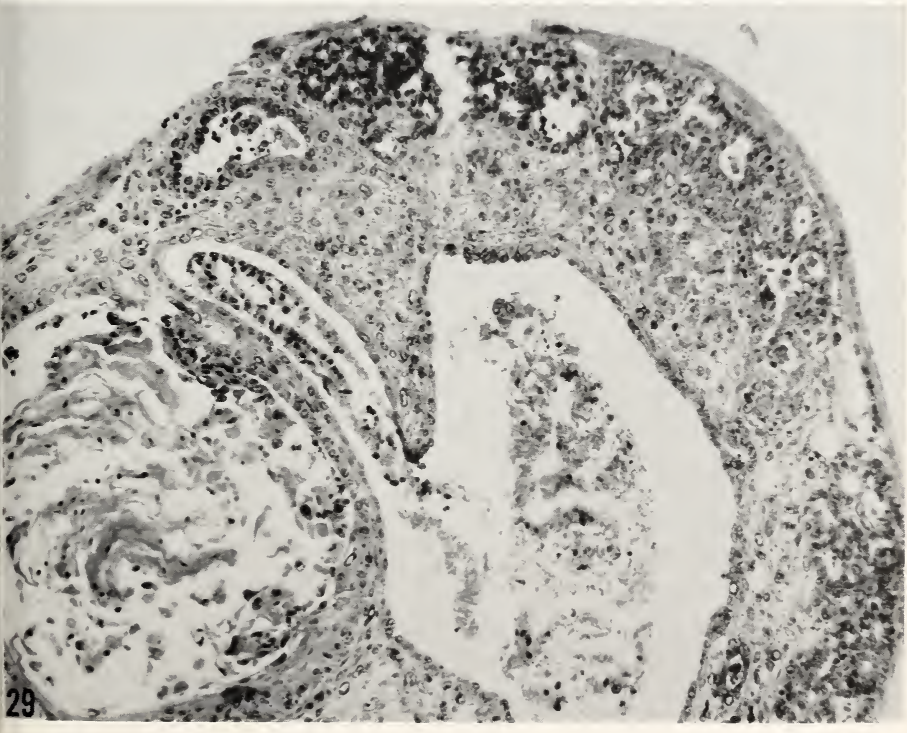

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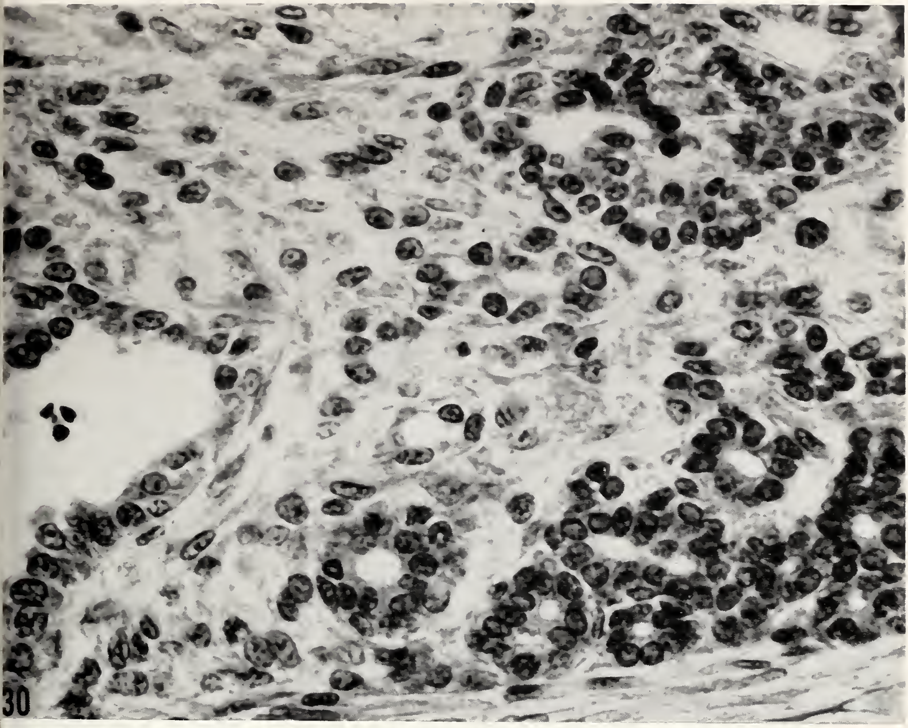
PLATE 21

FIGURE 30.—Higher magnification of area from section shown in figure 28. Viable epithelium and mesenchyme. Hematoxylin and eosin. × 610

FIGURE 31.—Higher magnification of area from section shown in figure 29. Necrosis of mesenchyme in *upper left*. Nucleus of mesenchymal cell *above* and to *right* of *center* contains irregular amphophilic bodies. *Compare* epithelial nuclear characteristics with those of epithelium in figure 30. Tubular pattern is partially disrupted. Portion of mitotic figure present in epithelial cell at *lower left*. Hematoxylin and eosin. × 610



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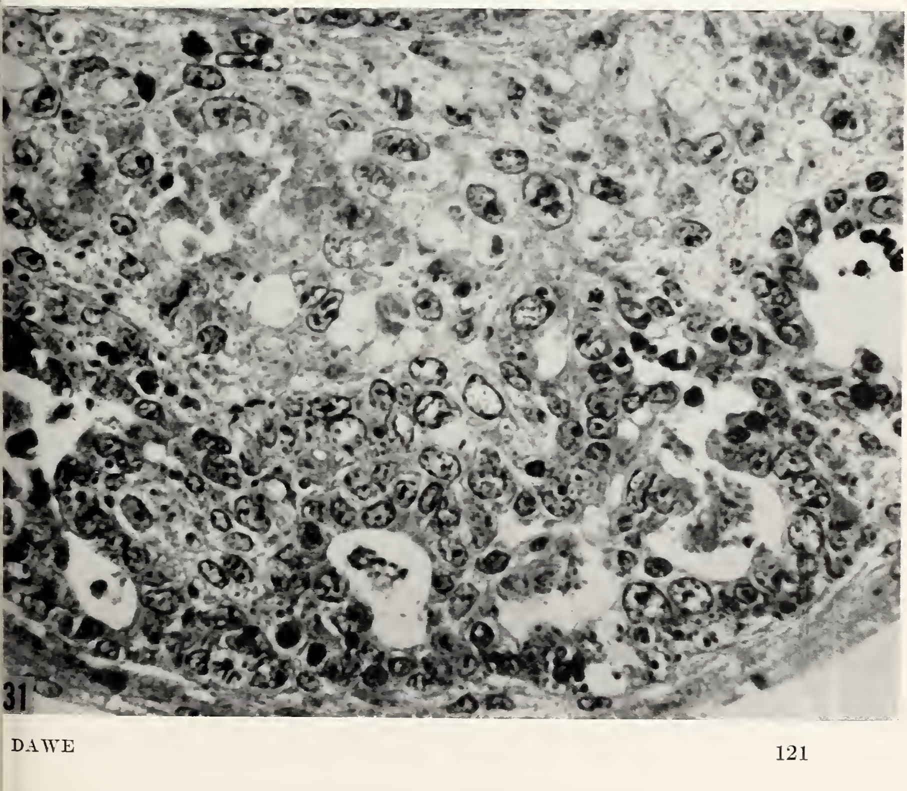

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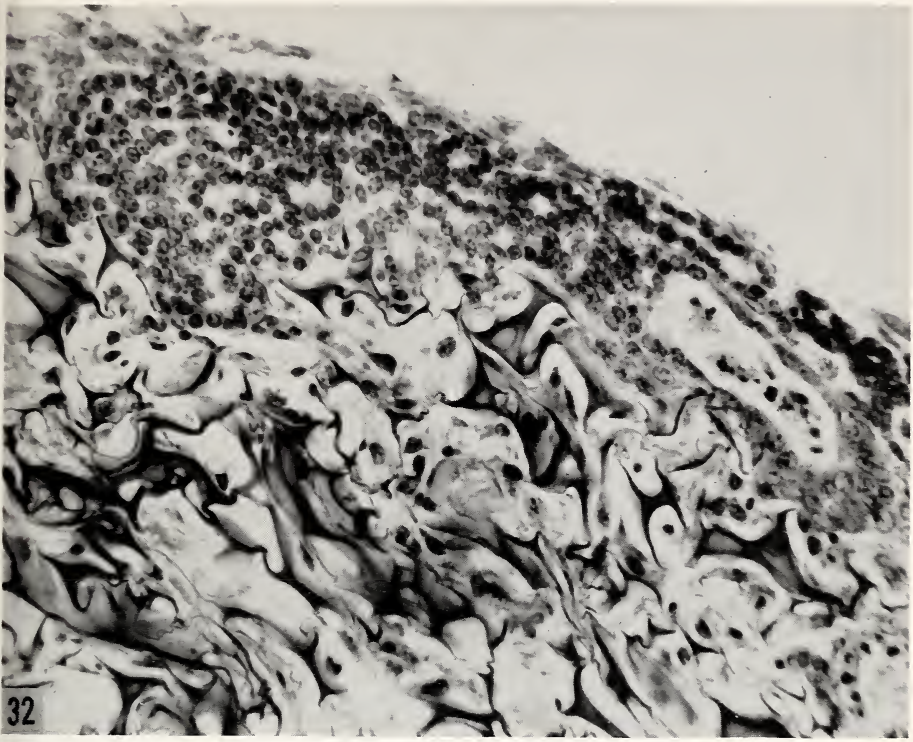
PLATE 22

FIGURE 32.—Uninfected gelatin-sponge control culture of 14-day submandibular rudiment 27 days after explantation. Portion of duct is on *right,* tubular epithelial structures on *left.* *Compare* with figure 33. Hematoxylin and eosin. × 420

FIGURE 33.—Culture similar to that shown in figure 32, but infected with polyoma virus at time of explantation 27 days previously. *Note* marked nuclear changes of ductal (*center*) as well as of tubular epithelium (*right*), with much necrosis in former. Squamous epithelium on *left* is probably from buccal epithelium included with rudiment during excision. Hematoxylin and eosin. × 420



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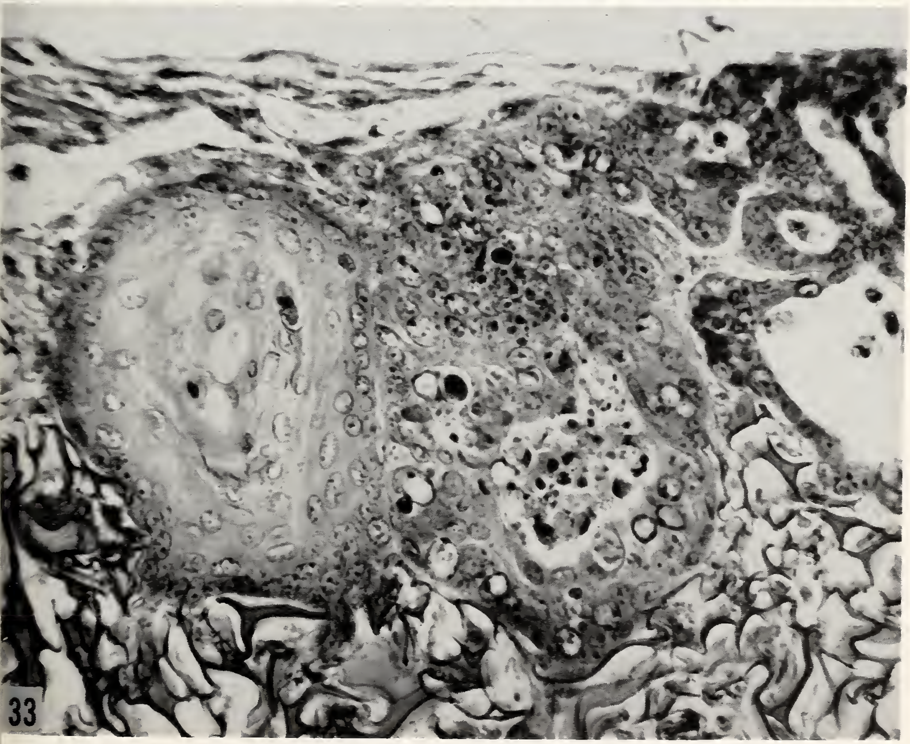

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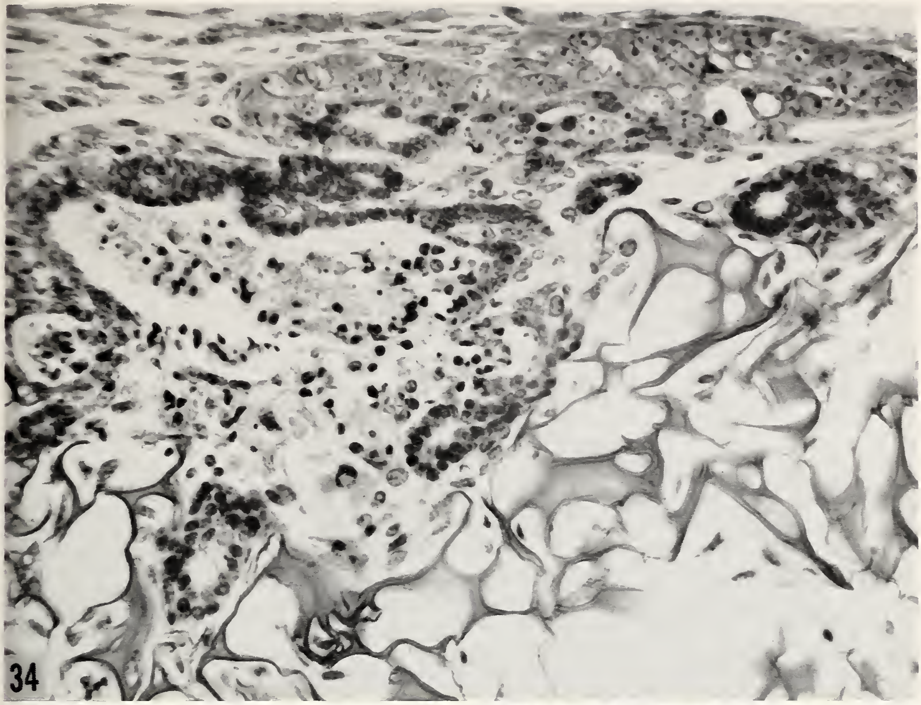
Plate 23

Figure 34.—Section of another gelatin sponge matrix culture of 14-day submandibular rudiment infected with polyoma virus and carried in culture for 27 days. *Note* (*top*) altered epithelium with large nuclei and nucleoli, and intranuclear vacuolation. Hematoxylin and eosin. × 420

Figure 35.—Higher magnification of altered epithelium in another area of section shown in figure 34. Mitotic figure is present among altered epithelial cells. Hematoxylin and eosin. × 1500



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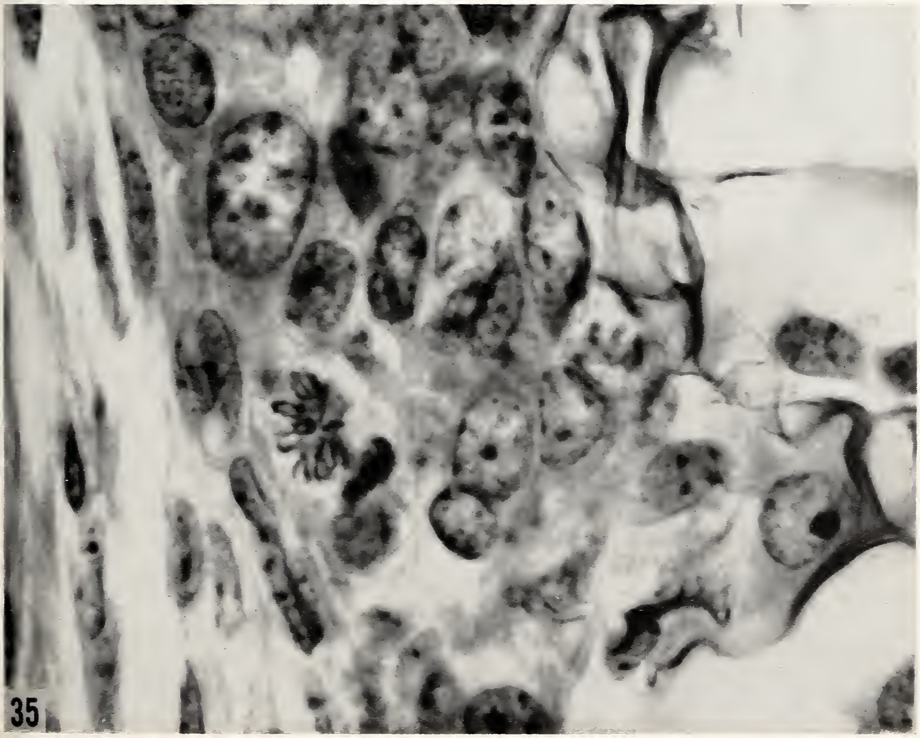

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Discussion

Dr. Andervont (National Cancer Institute): It was not clear to me whether Dr. Dawe implied that an important aspect of cancer is that the infected cell can supply something which will produce malignancy in an accompanying cell or whether the accompanying cell controls the activity of the infected cell.

Dr. Dawe: The concept is simply that the mesenchymal cell, as long as it remains in normal relationship with the epithelial cell, continues to exert its supportive influence on this cell and permits it to compete successfully with the virus, and even to proliferate. Perhaps proliferation becomes a necessity for survival under these circumstances. In an indirect way the mesenchymal cells would thus contribute to tumor development.

If this concept is correct, in order to provide conditions that would favor viral proliferation it would only be necessary to separate the previously infected epithelium from the mesenchyme. Under these conditions, the virus should then gain the "upper hand" and proliferate or perhaps complete itself, add a coat, and be released to infect other cells.

Dr. J. T. Syverton (University of Minnesota): We are indebted to Dr. Dawe for his careful guidance through the maize of polyoma-virus trophisms. Dr. Dawe has presented clearly his interesting findings. Two questions concern many of us: (*a*) What determines transition from tumor to cancer, and (*b*) what determines whether a virus results in proliferation or necrosis? Dr. Dawe, by using the submandibular gland as an organ culture to observe response to polyoma virus, has demonstrated that epithelial as well as mesenchymal cells respond to infection by hyperplasia. I interpreted the findings of Stanton and his associates, and those of Leuchtenberger, to indicate that polyoma virus exerts a necrotizing effect on epithelial cells and that the virus in turn either directly or quite indirectly provokes the mesenchymal cells to proliferate. Have you found by use of organ culture that you can separate proliferation of epithelial cells from cytolysis? Is mesenchymal proliferation independent of, or secondary to, epithelial proliferation? Do you find by use of organ culture natural attrition or loss of the mesenchymal effect?

Dr. Dawe: My impression is that the mesenchyme is not the proliferative part of this process, but the epithelium. The mesenchyme is the supporting element and must, according to this concept, be present or have exerted its influence possibly at some time prior to infection of the epithelial cell in order to make it capable of reacting with the proliferative type of response.

I am unable to interpret any of the proliferative response as being mesenchymal. When one deals with the hamster and other species, the question is entirely different, and I would not suggest that any such sort of concept that I have proposed could apply to the mesenchymal cells in the hamster, which apparently are the cells that respond. There are apparently some very different host-virus relationships concerned with the mesenchymal cells in that animal.

Dr. George O. Gey (Johns Hopkins University): Does this virus always give a necrotizing response?

Dr. Dawe: I presume you mean, Does it always give *some* necrosis? I would have to say that there *has* always been some necrosis. There has never been any pure proliferative response. This is a thing that is quite difficult to evaluate in tissue culture, since there is some necrosis in areas of many normal cultures. However, it is my impression, based on the nuclear lesions, that there is always some specifically virus-induced necrosis along with the proliferation.

Dr. Gey: When you get rid of the virus, what have you got? Here we have a disease which is produced in many organs and considered to be a neoplastic disease due to the polyoma virus. What are these cells in the hamster? Are they completely free of virus? If so, are they not new cells with a genome built into them which makes them different from what they were before?

I would like to try to delineate between noncytopathic and cytopathic change. Our experience does include tumor-producing viruses.

Dr. Andervont: If I understand Dr. Gey's question, he wants to know whether a cell that has proceeded to malignancy under the influence of the virus needs the continued presence of the virus to remain malignant. Is that the point?

Dr. Gey: Exactly.

Dr. Irena Koprowska (The Wistar Institute): Dr. Dawe, you have described intracellular inclusions. Can you compare them with the inclusions described by Leuchtenberger in tissue cultures infected with adenoviruses?

Dr. Dawe: I have not done any studies on the histochemical nature of the nuclear lesions. Dr. Love (National Cancer Institute) is presently doing some studies with the toluidine blue-molybdate method and the only thing that can be said is that the amphophilic inclusion, by his interpretations, contains RNA. The clear zone around this inclusion is much more difficult to interpret, and I do not believe Dr. Love is prepared at present to say what all is in there, so I must stop with that. I might add that Dr. Banfield (National Cancer Institute), who has done some electron microscopy on these cultures, has seen particles characteristic of the polyoma virus in areas that seem to correspond to the clear zones around the inclusions.

Dr. M. M. Sigel (University of Miami): I should like to suggest that there may be an alternate explanation, for the lack of cytopathogenic effect, to the one offered by the author, which is based on an interrelationship of mesenchyme and epithelium. This explanation may lie in the type of system used: the organotypic tissue culture.

It is well known that HeLa cells grown on glass in monolayers and exposed to poliovirus demonstrate cytopathogenic changes and disintegrate. In our experience, when the same cells are planted in the brain of an animal they show considerably less destruction or cytopathogenic change while still supporting growth of the poliovirus. Such cells form structural entities in the rat brain. It is therefore quite possible that structures, such as are retained in your cultures and that developed in the brains of our rats, may be a major factor in determining the fate of cells infected by virus.

Dr. Dawe: Yes, the interplay of mesenchyme and epithelium need not be a peculiar one in the sense that such a relationship might also exist for HeLa cells even in a heterologous host. It may be basically a nutritional relationship—a feeder-cell type of culture system. I do not know that your observation particularly contradicts this interpretation; it may parallel it to some extent. The same sort of thing might apply (some of the experts on Shope papilloma might have something to add here) to the Shope papilloma virus which is seen in its complete form in the outer layers of the epidermis rather than in the basal cells, in which the mesenchymal-epithelial relationship remains intact.

Dr. L. L. Coriell (South Jersey Medical Research Foundation): I would like to underscore the observation of the relationship between the epithelial cell and the mesenchyme in another system. HeLa cells in the normal rat grow well for a few days and then die, whereas in the cortisone-X-ray-treated rat, HeLa cells continue to grow until the death of the animal.

There is a striking difference between a normal animal, which discards the transplanted tumor cells, and the animal whose defensive mechanisms have been altered. In the latter there is little connective tissue or leukocytic reaction; the cells continue growing and the host provides a rich blood supply but no collagen formation.

Dr. W. R. Earle (National Cancer Institute): With a culture of very low cell density on glass with a fluid overlay, unless there is a certain specific cell density the cells will not live, or, if they live, they will show all sorts of disintegrative changes. When there is a mixed culture of epithelium and mesenchymal cells, is there a possibility that, instead of specific reaction between the mesenchyme and the epithelium, the mesenchyme destroyed the epithelium the way almost any type of cell would have done in order to survive?

Dr. Dawe: You are referring to the situation in which we isolated the epithelium completely and obtained a gradual death of the cells. This was one of the reasons that we took multiple epithelial rudiments and piled them up together, so that we would have approximately a cell mass about equivalent to what we had when we had an intact rudiment. Even under these conditions, the epithelium does not survive as long as it does when combined with mesenchyme.

Dr. Leon Dmochowski (The M. D. Anderson Hospital): I want to comment on the relationship of this virus to the kidney cells of the epithelial and connective-tissue origin. We have recently carried out a series of morphological investigations by means of the electron miscroscope in collaboration with Dr. B. E. Eddy and Dr. S. E. Stewart. We found that after the infection of mice, rats, or hamsters with polyoma virus, within 2 or 3 weeks the nuclei of epithelial cells (that is, of cells of the proximal and distal convoluted tubules) are filled with virus particles or viruslike particles. The cells are distinct by the appearance of these characteristic structures in their nuclei.

After the appearance of proliferative lesions in the epithelial elements of the kidneys of animals from the 3 species, the number of these characteristic structures decreases, and when tumors appear it is hard to find the viruslike particles in the nuclei of the kidney cells. Just before we came to New York, we found these characteristic structures in the nuclei of the cells of hamster kidney tumors induced by polyoma, but it took Dr. Gey about 3 weeks to find them. It appears therefore that only very few tumor cells contain these characteristic structures, but, during the initial course of infection, numerous cells contain these particles. The important question is, therefore, whether virus is necessary as a continuous stimulus of neoplasia or whether it acts only as an initiating stimulus.

Dr. Dawe: I would like to say something in reply to Dr. Gey's and Dr. Dmochowski's questions as to whether or not the virus has to be present in the cells after the tumor is established or whether the tumor can get along without the virus. In the oldest cultures that we have carried, only up to 75 days, we have a population made up entirely of altered cells. There are no unaltered cells left, but many of the altered ones show the inclusion bodies and the nuclear clear areas that are characteristic of the presence of virus. On the other hand, as Dr. Dmochowski has pointed out, in the fully developed tumors one does not see the nuclear lesions except occasionally at the margins of necrosis.

However, if one takes a piece of fully developed tumor, places it in a tube of medium, lets it drift there for anywhere from 1 day to 2 weeks, then removes it and examines it with the light microscope, one sees the appearance of the nuclear inclusions and the other nuclear lesions. Dr. Banfield, with the electron microscope, has found that many cells in such cultures contain typical polyoma-virus particles, whereas it is very difficult to demonstrate similar particles in tumors freshly removed from the animal.

Natural Factors Influencing Host Responses[1, 2]

O. MÜHLBOCK *and* **L. M. BOOT,** ***The Netherlands Cancer Institute, Amsterdam, Holland***

INVESTIGATIONS with the Bittner factor (the mammary-tumor agent or the milk factor) have aroused considerable interest ever since its discovery by Bittner (*1*). This agent, which behaves in many respects like a virus, is related to the development of mammary cancer, an epithelial tumor, in mice. The characteristic host response to this agent is therefore the development of mammary cancer at a late age in these animals.

This review considers the natural factors influencing host response to the mammary-tumor agent. The various aspects involved will be illustrated only with the investigations performed in the Netherlands Cancer Institute, Amsterdam.

Table 1 records the results of an experiment to demonstrate the presence of this agent. Susceptible female mice given the agent by foster-nursing have a high incidence (96%) of mammary tumors at an average age of 438 days. The controls have a low incidence of 3 percent at a considerable later age (700 days). The demonstration of the effect of the agent is as yet only possible by statistical means and after a long observation period—severe handicaps for the work with this agent.

TABLE 1.—Mammary-tumor incidence in (♀ O20 × ♂ DBAf)F_1 virgin females with and without the mammary-tumor agent

	Total No.	Mammary tumor (*percent*)	Average tumor age (*days*)	Average age at death without tumor (*days*)
With agent, foster-nursed by ♀ DBA	73	96	438	768
Without agent	93	3	700	786

This agent is instrumental only in the development of mammary cancer and does not influence the development of other tumors, as the results in table 2 show. Male mice which do not develop mammary

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[1] Presented at a Symposium on Phenomena of the Tumor Viruses, New York, N.Y., March 25 and 26, 1960.

[2] This investigation was supported in part by research grant C-3431 from the National Cancer Institute, National Institutes of Health, Public Health Service.

cancer were given a potent extract containing the agent at weanling age. The incidences of lung tumors and of hepatomas were recorded. Another group of the same mice were given an extract of mammary tumors without the agent. No significant differences in the tumor percentages were found, and the lifespan of these animals was not influenced by the presence of the agent.

TABLE 2.—Tumor incidence in (♀ O20 × ♂ DBAf)F_1 males with and without the mammary-tumor agent

	Total No.	Lung tumor (*percent*)	Hepatoma (*percent*)	Average age at death (*days*)
Untreated	94	16	1	672
With agent, given as extract from C3H tumors	91	8	3	664
Without agent + extract from C3Hf tumors	90	10	2	680

There are no obvious signs of the agent in the early life of the animals. Since the mammary-tumor agent is related to the development of mammary cancer, the first visible effects of the agent can be expected in the mammary gland. Hyperplastic nodules are generally considered to represent a precancerous condition. Hyperplastic nodules and carcinomas are also observed in agent-free mice, but the frequency of nodules and carcinomas is markedly increased in the presence of the agent. The influence of the mammary-tumor agent may be twofold: (1) The agent causes the development of more nodules, and the risk of development of carcinomas is proportionally increased. (2) The agent is associated with the development of more nodules, and these nodules show a more marked tendency to develop into carcinomas than they would in the absence of the agent.

From the experiments (*2–4*), in which the number of nodules was compared with the frequency of carcinomas in different strains of mice, with and without the agent, the following conclusion can be drawn (table 3): In the C3H and DBA strain, the nodules are numerous and the number of nodules is proportional to the mammary-cancer incidence. But in the O20 strain, the development of malignancy from these nodules is relatively more frequent. The reverse is true in the C57BL strain. Thus the odds that precancerous hyperplastic nodules in the mammary gland become malignant are different in various strains of mice, and depend primarily on genetically determined factors, which are independent of the presence of the mammary-tumor agent.

If this assumption is accepted, then the primary host response to the mammary-tumor agent would be the development of more hyperplastic nodules in the mammary gland at an early age.

There is no doubt that this response to the mammary-tumor agent is governed by genetic factors too. The number of the hyperplastic nodules

TABLE 3.—Frequency and tendency toward malignancy of hyperplastic nodules in the mammary gland

Strain	Agent present	Nodules	⟶	Carcinoma
C3H	+	++++	⟶	++++
C3Hf	−	++	⟶	++
DBA	+	+++	⟶	+++
DBAf	−	+	⟶	+
O20	+	+	⟶	++
C57BL	+	+	⟶	−

and the age at which they can be first observed are quite different in various inbred strains of mice. This brings up the question whether the mammary-tumor agent is the same in the various strains of mice; this is probably not the case (*5*, *6*). The agent of 5 different strains (C3H, DBA, RIII, WLL, and A) was administered to the same susceptible hybrids (♀O20 × ♂DBAf)F_1, either by the injection of extracts of mammary tumors or by foster-nursing (table 4). The agents of the first 4 strains gave a high incidence of mammary tumors, whereas the effect of the agent of strain A was quite different. Only a few mammary tumors developed in the test animals, though there can be no doubt that the A strain contains a mammary-tumor agent.

If the agent of the A strain is administered to different strains free of their own agent (C3Hf, DBAf, WLLf), the surprising fact is that it is effective only in the C3Hf strain (*6*).

TABLE 4.—Comparison of the host response to the mammary-tumor agent of different sources in (♀ O20 × ♂ DBAf)F_1 virgin hybrids

Sources of agent	Number of animals	Number with tumors	Average tumor age (*days*)
C3H	10	5	595
DBA	18	14	524
RIII	10	8	389
WLL	9	9	313
A	24	1	471

It may be concluded that the mammary-tumor agent from various sources differs in effect, either by a difference in quantity or, more probably, in quality.

Another experiment (*6*) showed that the agent of the A strain can enhance the effect of that of the C3H strain. Hybrids (♀O20 × ♂DBAf) F_1 were treated in the following manner: (1) One group was treated with the agent of the C3H strain at the age of 4 weeks; 50 percent developed mammary carcinoma. (2) Another group was treated with the C3H extract at 3 months of age; only 35 percent had mammary carcinoma. This confirms the long-known phenomenon that administration of the agent at a late age is less effective. (3) This group received the agent of the A strain at the age of 4 weeks; the mammary-tumor incidence was the same as in the control group. (4) Another group received the agent of the A strain when 4 weeks old, and the agent of the C3H strain 3 months later. This group showed the highest incidence of mammary cancer (88%). Thus there is an additive effect of the 2 agents.

Another factor that is genetically determined and that can influence the host response is the susceptibility to the mammary-tumor agent. Four different strains, which do not harbor the agent, were foster-nursed by the same strain (DBA). The virgin females of the IF strain had a mammary-tumor incidence of 100 percent; of the O20 strain, 30 percent; the CBA strain and the C57BL strain showed no mammary tumors.

As Gardner (7) showed many years ago, there is no doubt that the development of hyperplastic nodules is dependent on the presence of hormones. These are the same hormones that govern the growth of the mammary gland: hormones of the ovary (estrogen and progesterone) and the mammotropic hormone, prolactin, of the anterior lobe of the pituitary gland.

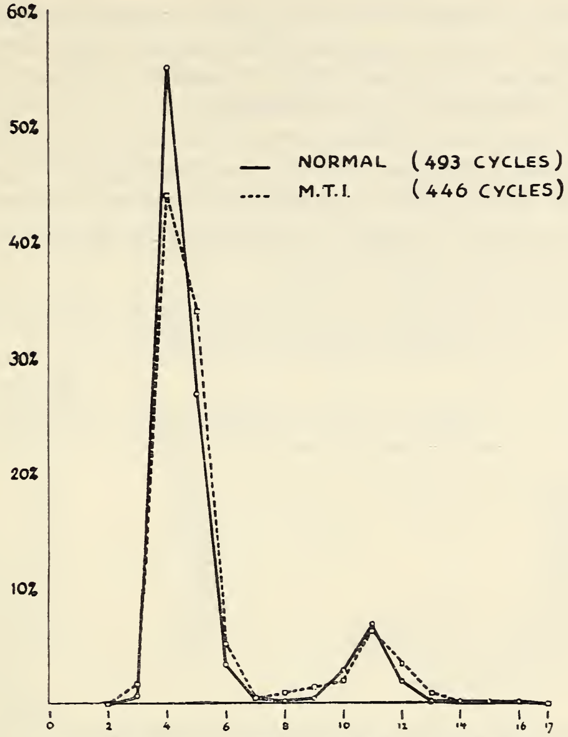
The experimental evidence indicates that the amount of hormones acting is important. Generally it may be stated that a higher dose enhances the development of the nodules more effectively. It has long been known that pregnancy, too, shows the same effect. Pregnancy is a complex phenomenon, but there are reasons to assume that the pregnancy effect is due to a stronger hormonal influence on the mammary gland.

Two possibilities for the mechanism of action of the mammary-tumor agent can be considered: (1) The agent acts via an influence on the hormone production or the hormone balance. (2) The agent influences the susceptibility or reactivity of the mammary gland to the hormonal influence.

To investigate the first possibility, the effect of the mammary-tumor agent on the estrous cycle was determined. Forty-seven animals of the agent-free hybrid (♀C57BL × ♂DBAf)F_1, known to be susceptible to the agent, received intraperitoneal inoculations at the age of 4 weeks of extracts of mammary tumors of the C3H strain. Three months later daily vaginal smears were taken for a period of nearly 2 months. Forty-nine untreated animals of the same hybrid served as controls.

The length of the estrous cycle was determined as shown in text-figure 1. During the experimental period, the controls showed 493 complete cycles and the treated animals 446 cycles. The duration of the cycles varied in both groups from 3 to 16 days, as calculated from estrus to estrus. In both groups the large majority of the cycles had a duration of 4 to 6 days. There was no difference between the animals with the mammary-tumor agent and the controls in the average duration of these short cycles.

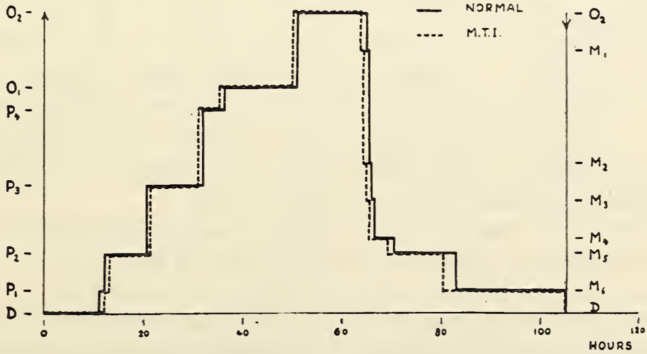
The second group of cycles (duration from 9 to 16 days) formed 14 percent of the total number of cycles in the treated animals as against 13 percent in the controls. The prolongation observed was always due to a lengthening of the diestrous periods; this group of cycles constitutes the



TEXT-FIGURE 1.—Length of estrous cycle in days in (C57BL × DBAf)F_1 mice.

so-called spontaneous pseudopregnancies, in which functional corpora lutea are present, which indicate prolactin secretion by the pituitary. We must thus conclude that the mammary-tumor agent has no significant effect on the incidence of these pseudopregnancies, *i.e.*, on prolactin secretion.

We next determined the duration of the various stages of the estrous cycle in the animals with the mammary-tumor agent and the controls (text-fig. 2). Here too we failed to observe any difference between the 2 groups.



TEXT-FIGURE 2.—Length of various stages of the estrous cycle in (C57BL × DBAf)F_1 mice.

In summary, we can conclude that this experiment failed to produce any indication that the mammary-tumor agent affects either pituitary function in respect to gonadotropin or prolactin production, or directly or indirectly the ovarian hormonal function.

The following evidence will, however, show that the phenomenon of spontaneous pseudopregnancy has a direct bearing on the question of the ultimate effect of the mammary-tumor agent, if only in a rather negative sense. The incidence of these pseudopregnancies appears to vary greatly between strains, as is shown in table 5.

TABLE 5.—Spontaneous pseudopregnancy in various inbred strains of mice and F_1 hybrids*

Strain	Number of cycles studied	Pseudo-pregnancies (*percent*)
IF	105	44
C57BL	115	23
DBAf	116	27
O20	186	14
C3Hf	332	12
CBA	282	11
A	329	4
(♀ O20 × ♂ DBAf)F_1	312	12
(♀ C57BL × ♂ DBAf)F_1	939	14

*Four animals per cage.

The incidences thus vary between 4 and 44 percent. Of special interest is the occurrence of only a low percentage in the mammary-tumor-agent-containing strain A. This strain is exceptional in another respect: It is the only agent-containing strain in which we can observe a low incidence of mammary carcinoma in virgin animals and a much higher incidence in animals that have been made pregnant or pseudopregnant only a relatively small number of times. This phenomenon can occur only in a strain with a normally low number of spontaneous pseudopregnancies, as otherwise induced pseudopregnancies could not be expected to have such a marked influence.

If the agent does not influence the hormone production, it would follow, *per exclusionem*, that its effect has to be sought in an influence on the susceptibility or reactivity of the mammary gland. Experimentally this is difficult to prove. No effect of the agent could be found in young animals in tests for the reactivity of the mammary gland to estrone (table 6). It is probable that the agent acts only after a certain latency period. Thus far the only visible effect of the agent is the development of hyperplastic nodules in the mammary gland.

It is conceivable that the host response can be influenced by factors which act on the propagation and transmission of the agent.

There are strains of mice that propagate the mammary-tumor agent and transmit it from generation to generation continuously, and there are strains that cannot propagate the agent continuously and thus can be considered as naturally agent-free. A proper genetic constitution is thus the premise for the propagation and transmission of the agent. Little is known of how this mechanism acts.

The question can be raised whether the capacity to propagate the mammary-tumor agent and transmit it from generation to generation continuously is correlated with susceptibility to the development of mammary cancer after hormonal treatment only. For this reason, we compared the mammary-tumor incidences after standardized hormonal treatment in two agent-free sublines of strains that naturally possess the agent (C3Hf and WLLf), one strain (IF) which can propagate the agent, without, however, being infected naturally, and three strains (O20, CBA, and C57BL) which cannot propagate the agent continuously from generation to generation and thus are naturally agent-free.

TABLE 6.—Threshold dose of estrone for growth of mammary gland in castrated mice with and without the agent

Strain	Estrone (μg.)				
	0. 01	0. 05	0. 1	0. 2	0. 3
♀ DBA (with)			−	(+)	+
♀ DBAf (without)			−	(+)	+
♂ DBA (with)			−	(+)	+
♂ DBAf (without)			−	(−)	+
♀ A (with)	−	+	+		
♀ Af (without)	−	+	+		
♀ C57BL (with)	−	(+)	+		
♀ C57BL (without)	−	(+)	+		

As hormonal treatment we chose the most effective method known: the isografting of pituitaries in otherwise intact female animals. The transplants produce prolactin continuously and thus induce a hormonal constellation which favors mammary-gland carcinogenesis. The results are shown in table 7.

TABLE 7.—Mammary-tumor frequency after subcutaneous isografts of 20 pituitaries in inbred strains of mice without the agent

Strain	Number	With tumor		Without tumor
		Percent	Average tumor age (*days*)	Average age at death (*days*)
C3Hf	18	89	419	386
WLLf	50	18	622	583
IF	19	0	—	477
O20	40	67	531	548
CBA	14	50	527	576
C57BL	32	12	454	583

The conclusion must be that a correlation between the susceptibility to the development of mammary cancer after hormonal stimulation and the propagation and/or transmission of the agent does not exist.

The limited data available suggest, however, that there does exist a correlation between the susceptibility to develop mammary carcinoma after hormonal treatment and the susceptibility to develop these tumors after administration of the agent. Strains with a high incidence of mammary carcinoma in the presence of the agent generally show also a high incidence of these tumors in the absence of this agent, after suitable hormonal treatment.

In strains which possess a high susceptibility to mammary carcinoma after either hormonal treatment, *i.e.*, implantation of pituitaries, or after infection with the agent, it is difficult to show clearly the interaction after combined treatment. The C57BL strain, however, is suitable for the demonstration of this interaction. Hormonal treatment alone or the mammary-tumor agent alone induces but a few tumors in otherwise untreated female animals of this strain. Combined treatment induces a high incidence of mammary tumors, as is shown in table 8.

TABLE 8.—Mammary carcinoma in C57BL female mice after various treatments

		With tumors		Without tumors
Treatment	Number	Percent	Average tumor age (*days*)	Average age at death (*days*)
Controls (virgins)	193	0	—	621
Fostered on (♀ C3H × ♂ WLLf) F_1 (virgins)	61	11	507	602
20 Pituitaries, subcutaneous	32	12	454	583
Fostered on (♀ C3H × ♂ WLLf) F_1 and with 20 pituitaries, subcutaneous	25	56	460	491

From the evidence presented, the host response to the mammary-tumor agent, *i.e.*, the development of mammary cancer, is determined primarily by hormonal factors. Thus the host response is dependent not only on factors directly or indirectly acting on the mammary-tumor agent as such, but also on those factors acting on the endocrine system.

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Discussion

Dr. Andervont (National Cancer Institute): Probably the best point you made, Dr. Mühlbock, is that here is a virus which is not the whole story in the production of malignant cells. There are other factors that must be present before it can function. And this, of course, is provocative.

Dr. W. U. Gardner (Yale University): On this occasion I think that we could appropriately mention the late Dr. Leo Loeb who about 40 years ago first proposed a simple formula for mammary cancer in mice—genes plus hormones give cancer. This is doubly appropriate today because Dr. Mühlbock has stated that the transplantation of pituitary glands abets mammary tumorigenesis, even in mice without identifiable mammary-tumor agent. Dr. Loeb and his associates first showed, about 21 years ago, that, in mice showing a low tumor incidence, the incidence of mammary tumors could be greatly increased by implanting one, two, or three pituitary glands, even when they could not find the transplants at necropsy. Dr. Mühlbock's observations have revealed the importance of proper hormonal conditions in mammary carcinogenesis in mice and thus added evidence to the formula.

Our early work indicated the importance of a maternally transmitted influence on the appearance of multiple localized hyperplastic nodules in the mammary glands of female mice or of mice under adequate hormone treatment. These nodules appeared in large numbers in the mammary glands of older multiparous mice of the C3H and DBA strains which showed a high incidence of spontaneous mammary tumors. They increased in number with age and decreased with the postlactation interval. The glands of the high tumor mice of strain A showed fewer nodules but the ducts more frequently gave origin to tumors. Nodules were lacking in comparable mice of the low tumor N strain. We believed these nodules to be induced by the Bittner agent after this agent was defined biologically. But mammary nodules are of two types: (1) those that represent localized areas that do not regress as rapidly as adjacent areas (retention type), and (2) those that represent localized proliferation of otherwise quiescent mammary tissue. The retention type disappears during the postlactation period. Qualitatively the two types of nodules may be difficult to distinguish between in gross preparations. We assumed that the agent promoted growth of the localized hyperplastic nodules in a suitable hormonal environment and that other influences determined their neoplastic transformation.

Hypophysectomized mice quickly show regression of these nodules for the greater part. A few, not over 5 percent, persist and these probably were already adenocarcinomas at the time of hypophysectomy. Mice given testosterone propionate will also show very few of the local hyperplastic mammary nodules, even when estrogen is given, and will acquire few tumors. After nodules have formed, the injection of testosterone will not prevent mammary cancer or the persistence of some nodules, though it does reduce their number.

Genetic influences are also of importance in mammary carcinogenesis in mice. Hybrid offspring of a low mammary tumor strain (C57) and a high mammary tumor strain (CBA) give F_1 hybrid mice, which tend to show an incidence of mammary adenocarcinomas that parallels the maternal CBA strain and mammary tumors of a squamous type occur at a lower incidence among the reciprocal hybrids. The adenocarcinomas contain agents, the others do not. The backcrosses of the F_1 hybrids to the parental strains indicated that the C57 genotype, even in the presence of the Bittner milk influence, resulted in few mammary tumors. This was also revealed by other hybrid crosses. In all these experiments, the agent was transmitted through the milk by nursing rather than injected or fed after concentration from milk or some other source.

We must recall that mammary tumors have been induced under a variety of conditions in mice: (1) known agent + hormone + adequate genotype, (2) adequate genotypes and hormones (pituitary and gonadal) — known agent, and (3) hormones and carcinogenic hydrocarbon — known agent. All must act over comparatively prolonged periods before mammary cancers appear. It seems to me that we must consider the possibility either (1) that we are dealing with more than one type of disease showing similar symptoms, or (2) that all the known mammary carcinogenic influences act through a common causal agent, possibly with the formation of a causal agent or "iso-carcinogen" in the mammary tissue itself.

Dr. Beard: It would seem appropriate to comment on the influence of the host in the occurrence of the malignant process associated with virus infection. To me it would be impossible to have any understanding of the contribution of the host, qualitative or quantitative, to viral response without some idea of the mass factors concerned with the material used for the transmission of the diseases.

Twenty years ago we were greatly impressed with the work of Dr. Ray Bryan which showed that the ID50 dose of the Shope papilloma virus was about 80 million particles in domestic rabbits. Such a relationship has been observed with all the virus tumors thus far studied on a quantitative basis. In myeloblastosis, the ID50, measured in a highly susceptible strain of chickens, is about 24 million virus particles and in erythroblastosis the comparable infectious unit is 24 thousand particles. It is thus quite evident that, although the virus determines the cellular characteristics of host response, it is largely the chicken or the rabbit which determines whether or not disease will occur. Many strains of chickens hardly react at all to either avian virus, but some respond with high incidence.

In relation to mouse mammary carcinoma, there has been much study of the relative influences of hormones, virus, and genetic constitution. The truth is that what has been needed, though yet lacking, in the study of mouse mammary carcinoma, as well as other virus tumors and tumor viruses, has been the correlation of the various concepts as to the variety and the degree of influence of the factors which can contribute to the processes of induction and maintenance of malignancy.

I do not have the slightest doubt that mice may develop carcinoma of the breast without the participation of virus, and this might very well be influenced to a certain extent by kind and quantity of hormone. In contrast, it was disappointing to hear the serious consideration given to the possible influence of the virus on the production of hormone. While such a concept seems entirely remote, there would be no difficulty in understanding that, on the other hand, the hormone might well influence the preparation of breast tissues which would make possible reaction of the cells to the virus.

In principle, viruses are much like bacteria, with the difference that bacteria live outside cells and viruses exert their influence and are synthesized intracellularly. Everyone knows that bacteria cannot grow without proper media and some of these organisms, like tubercle bacilli, have very special requirements. If this is the case with bacteria, how could one suppose that it would not be even more necessary to have a responsive medium inside the cell for viruses, whose requirements are so much more specific than those of bacteria? As far as I am concerned, the matter with respect to the mouse mammary carcinoma is relatively simple. There exists a specific viral agent which has the capacity to induce specific malignant reaction in the cells of the mammary gland. Without the proper conditions for the growth and the propagation of the virus inside the cells, no response is observed. Given the opportunity for proper conditioning of the physiological state, however, the cell develops the capacity to respond to the virus, and carcinoma arises.

Analogous phenomena are observed throughout the whole tumor virus field. Many different kinds of factors determine whether or not a cell can respond to a virus which may have been in cells from the very beginning of the life of the individual. It is clear that the genetic constitution of the mouse is a critical basic factor. In addition, the painful investigations of many workers have revealed the unquestionable influence of hormones. Yet, except for the establishment of a limited number of qualitative principles, the results of such work have actually advanced the total problem but little toward the solution of the question of the intimate cell-hormone-virus relationships involved in the induction of malignant change. If judgment can be made from the experience with a few other virus tumors, such as, for example, those of the bird, it can be assumed with assurance that significant progress with the mouse mammary carcinoma will be dependent eventually on quantitative correlations of the respective influences of virus and hormones. The critical unknown thus far is the virus. In consequence, regardless of the cost or difficulty, further elucidation of the processes leading to the onset of mouse breast cancer will not be made until the agent can be quantitated physically and biologically.

Dr. Andervont: Since Dr. Beard has told us so eloquently that the problem of breast cancer in the mouse is very simple, I am calling on someone who I deem has had sufficient experience also to tell us how simple it is, Dr. John Bittner.

Dr. John J. Bittner (University of Minnesota): Well, this has been very, very interesting. For about 5 years, this gentleman has been telling us that mammary cancer is a very, very simple problem. A few years ago I asked Ray Bryan, who worked on the mammary-tumor agent, and published in 1944, why he did not come back and clear up the mess which Dr. Beard says we are in. And Ray said, "John, I would not live long enough to do so."

I think, after listening to Dr. Mühlbock, that everyone who has worked on mammary cancer in mice is in agreement as to the role of the agent, hormones, and susceptibility. The only possible difference of interpretation could be explained by a difference between the incidences of the different strains maintained in Europe and in this country. Otherwise, the interpretation he has presented would be similar to that of others who have worked on the disease.

He said that, if the agent from five different strains was introduced into one host strain, there were differences in the incidence; of the stocks which he tested, strain A had a very inactive agent.

In this country Andervont and Heston have compared strain A and the C3H strain. There was no difference in the incidence of tumors in the reciprocal F_1 hybrids. When we, using our strains, compared the reciprocal cross between strain A × C3H, those with mothers from strain A had a higher incidence than those with C3H mothers. In our hands the agent from strain A was more active than the agent from strain C3H.

When we made reciprocal crosses between the A, C3H, and DBA strains, in the A × C3H cross the strain A agent was more active. In the C3H × DBA, the C3H agent was more active, and in this cross the *C3H agent* was more active than the *A agent* in the A × C3H cross. In strain A × DBA, the A agent was more active, but when we crossed the DBA and the C3H, the DBA agent was more active than the A agent in the A × DBA cross. So you can take your pick, for the activity of any agent will be influenced by the constitution of the host.

I think that Dr. Mühlbock might give more consideration to the role of the adrenal in the development of mammary cancer. We have confirmed his work on the pituitary implants by inducing tumors in agent-free mice in which we cannot demonstrate the agent. In certain of our strains we can induce mammary cancer by implanting three pituitaries. Dr. Mühlbock has been able to induce mammary cancer by implanting one pituitary.

Dr. Halberg has obtained evidence that in mice with pituitary implants there is an increase in the serum adrenal corticosterone. In other words, he can pick up a difference in hormonal level in mice, after the implantation of pituitaries, that can be detected within 3½ months.

Now, supposedly the pituitary is under the control of the hypothalamus. If pituitaries and a hypothalamus are implanted at the same site the development of mammary cancer is inhibited, not only in a low cancer strain, but in mice with the agent.

If the pituitary is implanted on one side and the hypothalamus on the other side of the mouse, the growth of the mammary cancer is accelerated.

If mice are painted with methylcholanthrene, mammary cancer is induced in some agent-free strains. If mice of the same strain are used with the agent, and 95 percent will have mammary cancer as either virgins or breeders, the incidence is reduced by painting them with the same hydrocarbon. Here is a compound which will induce mammary cancer in agent-free mice or inhibit mammary cancer in agent-containing mice.

Personally, I would like to see Dr. Beard work with mammary cancer in mice. He talks about the increase in incidence with the administration of an increased number of virus particles. When mammary tumors are assayed, the same extract will produce many more tumors when a 10^{-4} dilution rather than a 10^{-1} dilution is used.

It is regrettable that this does not fall into line with the chicken tumor virus. All we are doing is reporting facts as we see them, not as we would like to have them. If theories would solve the cancer problem, it would have been solved a good many years ago, but there is still a great deal of work to be done. In 1947 I was a coauthor on a paper suggesting that the role of the agent in the induction of mammary cancer in mice was to alter the hormonal metabolism, and I still believe that is the case—that the hormones become the primary inciters for mammary cancer in mice. If some investigators say we have an agent in these tumors that we cannot demonstrate, I feel it is up to them to show us how to do it. They can certainly get mice from us; I am sure that all of us will supply them with mice and they can work with their own animals in their own laboratory. They may survive longer than Dr. Bryan and solve the problem.

Dr. G. Miroff (University of North Dakota): Many years ago in Dr. Bittner's laboratory he related these complex situations to me. I was convinced that there must be a simpler way to test for the agent than by the protracted bioassay.

After a series of studies, I came to the conclusion in a roundabout way that it might be possible to show the effect of the agent in mice which were within the age of greatest susceptibility. The livers of mice, suckling on mothers with and without the agent, and the milk or milk fractions, when separated on substituted cellulose columns and then injected into suckling mice without the agent, produced a differential effect in the liver phospholipides. The interesting thing is that this biochemical difference disappears when the mice cease breast feeding, which indicates that the agent may insult the tissue and become integrated or incorporated into the metabolism or structure of the cell. This alteration, through hormones or in some other way, may then produce tumors only after the breast tissue has developed.

Dr. Mühlbock: I am glad that two speakers have supported my views. When I accepted the invitation to attend this meeting a few months ago, I talked with Dr. Beard, and he said, "I will give you hell." So I did not know what would happen to me.

I think the situation is quite clear. I am not a virologist; indeed, I do not know much about virology. I have applied half my lifetime to the mammary cancer of mice and to hormones, and Dr. Beard has stated he does not know anything about mammary cancer, but I think he knows about virus particles.

I could say, "What is the sense of my coming here and talking?" I think it is very sensible indeed, because we have to get together and learn from each other so that we will be prevented from looking at only one angle of the cancer problem. Then we learn that there is not only one single problem but many to be solved. As a nonvirologist in a meeting consisting mainly of virologists, I would like to stress this point: There are other factors which are instrumental in the development of cancer. If we keep this in mind, I think sometime in the future we will come together and say, "Now, we agree altogether."

Experimental Alteration of Host Response to an Occult Tumor Virus: Induction of Thymic Lymphosarcomas in X-Irradiated Mice[1,2]

HENRY S. KAPLAN, *M.D., Department of Radiology, Stanford University School of Medicine, Palo Alto, California*

In the past several years it has become well established that the lymphosarcomas and lymphatic leukemias (collectively termed lymphomas) which arise "spontaneously" in mouse strains AK and C58 are due to a virus. The agent is transmissible by cell-free filtrates (CFF) and centrifugates (CFC) which, when inoculated at birth, elicit the same kind of neoplasms in mice of normally nonsusceptible strains (*1*) and greatly accelerate their development in mice of the strain of origin (*2*). The presence of the thymus is essential for tumor development in filtrate-injected mice (*3–5*). Serial passage of filtrable material from tumors with short latent periods significantly increases the activity of the agent, as judged by greater tumor incidence, progressively shorter latent periods, and capacity to induce tumors when the agent is inoculated into older mice (*6, 7*). Electron micrographs of ultrathin sections of such filtrate-induced lymphomas reveal characteristic particles in the cytoplasm or in the extracellular spaces adjacent to the plasma membrane of thymic tumor cells (*8*).

Meanwhile it had long been known that lymphomas may be induced in very high incidence in normally nonsusceptible strains of mice by exposure to certain exogenous carcinogens (*9*). The morphologic identity of the virus-induced and carcinogen-induced lymphomas suggested the possibility that the action of the carcinogens in the inducible strains might also be mediated by an occult virus. Such an induction sequence might be imagined if the carcinogen were, for example, able to suppress "carrier" host resistance, or to release the virus from a bound intracellular site, or both. This possibility has now been investigated for one external agent, X irradiation. While the evidence still leaves much to be desired, the data from work with different strains in different laboratories are at least strongly suggestive. I shall limit my presentation to a summary of this evidence and to some tentative interpretations which are put forth to stimulate further discussion and experimentation.

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[1] Presented at a Symposium on Phenomena of the Tumor Viruses, New York, N.Y., March 25 and 26, 1960.

[2] Previously unpublished work cited herein was supported by grant C-3352 from the National Cancer Institute, National Institutes of Health, Public Health Service.

Most of the pertinent work has been done with strains C57BL, BALB/c, C3H, and their reciprocal F_1 hybrid crosses. "Spontaneous" lymphoma incidence in untreated mice of these strains is essentially zero up to 18 months of age, rising to perhaps 0.5 to 4 percent in older mice. The lymphomogenic efficacy of X irradiation is most striking in strain C57BL mice, in which, under optimal experimental conditions, a tumor incidence of between 90 and 100 percent is regularly attainable (*10*). The first deaths due to induced thymic lymphomas in this strain occur at about 90 to 100 days after X-ray exposure, and incidence reaches a plateau by 8 to 12 months. Strains BALB/C and C3H are somewhat less susceptible to comparable doses of X radiation (*9, 11*).

CFF and CFC, prepared from radiation-induced C57BL thymic lymphomas by the method of Gross (*1*), have been inoculated into isologous and F_1 hybrid mice at birth. Controls have included untreated mice of the same genotypes and mice inoculated at birth with heated (56°, 30 min.) lymphoma CFF, CFF from normal thymus, and CFF from other, nonlymphomatous neoplasms of isologous origin. Filter checks and transplantation characteristics of CFF-induced lymphomas have been used to exclude tumor-cell contamination of the filtrates and centrifugates. The observed lymphoid tumor yield from fresh lymphoma CFF, though very significantly higher than that in any of the control groups (*12*), still falls far short of the incidence attainable in irradiated mice of these strains, and the onset of these lymphomas is late.

Serial passage of CFF from filtrate-induced lymphomas through newborn mice results in a very appreciable augmentation of tumor incidence and reduction of latent period (*12*). Similar results have been independently obtained with CFF from radiation-induced lymphomas arising in strain C3H mice (*13, 14*). Heated lymphoma CFF and CFF from normal thymus and other types of neoplasms were inactive, whereas CFF prepared from a long-established transplantable lymphoma, originally radiation-induced in the thymus of a strain C57BL mouse, was as active as CFF from freshly induced lymphomas (*12*). In one experiment, CFF prepared from a virus-induced lymphoma of strain AK mice was inactive in strain C57BL mice inoculated at birth (*12*), which suggested that the agent in strain C57BL lymphoma CFF, though perhaps closely related, was not identical with the lymphoma virus of strain AK. In another experiment,[3] CFF from radiation-induced C57BL lymphomas failed to elicit tumors after inoculation into newborn C3H mice. Attempts to date to demonstrate viruslike particles in radiation-induced thymic lymphoma cells by electron microscopy have been generally unsuccessful (*15*), though such particles have been found in the extracellular spaces of lymphomas induced in C3H mice by CFF prepared from X-ray-induced C3H lymphomas.

To recapitulate, then, the evidence indicates that (1) an agent is present in CFF and CFC prepared from initially radiation-induced mouse lymphomas; (2) this agent can induce identical lymphomas in nonirradiated susceptible mice inoculated at birth; (3) the activity of the agent is appreciably stepped up by serial passage; (4) activity is destroyed by mild heating; (5) normal tissues and other types of neoplasms do not contain demonstrable amounts of the agent; and (6) the agent derived from one strain of mouse is tentatively believed to be specific for mice of that strain or their F_1 hybrids. It would be difficult to imagine a chemical agent that is extractable from a specific tumor, is then capable of inducing the same type of tumor, is extractable in increasingly greater yield on serial passage, and exhibits host strain specificity. By exclusion, therefore, the agent is best interpreted as being a virus or viruslike self-replicating genetic determinant. Progress toward the isolation, purification, and further characterization of the agent is seriously hampered by the lack of a rapid and sensitive assay method, preferably in an *in vitro* system, such as has recently been developed for the Rous (*16*, *17*) and polyoma (*18–20*) viruses.

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[3] Kaplan, H. S. Unpublished.

Although efforts to develop such an assay are the most urgent need at this time, other kinds of experiments can also contribute to our understanding. Among the questions we may ask are: (1) What is the essential lymphomogenic action of X radiation, or put differently, why is it necessary to irradiate mice of certain strains (C57BL, BALB/c, and C3H) and not others (AK and C58)? (2) Is the profound inhibition of lymphoma development in marrow-shielded or marrow-injected C57BL mice (*21*) due to restoration by the marrow of radiation-injured immune mechanisms? (3) Is the influence on lymphoma incidence of the hormonal environment (*22*) consistent with what we know of virus growth requirements generally? (4) Are occult viruslike agents also involved in lymphoma induction by other carcinogens, such as methylcholanthrene or estrogen, and if so, how do these filtrable agents relate to those extracted from radiation-induced lymphomas? It now seems possible to answer question 2, and to make some tentative suggestions about question 1; the other questions remain to be explored.

In a recent experiment, C57BL mice were exposed to X irradiation with or without lead shields over one thigh. The radiations were given in 2 courses 1 month apart, each course consisting of 2 weekly exposures of 168 or 200 r. Thigh-shielded irradiation is known to yield a much lower lymphoma incidence than unshielded whole-body irradiation; this effect is apparently mediated by bone marrow cells protected by the lead shield and can be reproduced by injection of compatible marrow cells from other donors into unshielded mice (*21*). Since whole-body X irradiation, particularly at higher dose levels, is known to render animals more susceptible to bacterial (*23*) and viral (*24*) infection, one ready explanation for the thigh-shielding effect is that it promotes early recovery of immunity in general and of immunity to the lymphoma virus as a by-product. If this were true, one would expect that an initial course of thigh-shielded irradiation would reduce the effectiveness of a second course of unshielded radiation given 1 month later. In fact (*25*), lymphoma incidence in such a group was significantly *greater* than that of animals given the second unshielded course alone, and essentially identical with that of animals exposed to unshielded radiation in both courses. The nature of the "priming" effect produced by the first shielded course is not clear, but the result cannot be reconciled in terms of the immunity hypothesis. This conclusion is also supported by some published work of Uphoff and Law (*26*), who found that injection of fetal hematopoietic tissue, which should not be immunologically competent, conferred almost the same degree of protection as did adult marrow cells against lymphoma development.

Microscopic examination of C57BL thymus glands at serial intervals after irradiation reveals an early, vigorous regeneration and differentiation of the cortical lymphoid-cell elements in marrow-shielded animals and an apparent maturation delay, associated with a persistent abundance of large, immature lymphoblastic cells, in the unshielded mice, which, it will be recalled, are destined to develop a high lymphoma incidence. It may therefore be much more than an interesting coincidence that the thymuses of normal newborn mice, which are highly susceptible to CFF-induced lymphoma development, also exhibit almost a "pure culture" of such large lymphoblastic cells in the outer half of the cortex. If one assumes that these two observations are indeed related, they may indicate a preference by the virus for proliferation in host cells that are at a particular stage of lymphocytic differentiation. Such specificity of appetite has been noted in a few instances with non-tumor viruses (*27*).

If, as a result of insult produced by radiation, transplantation (*28*, *29*), or urethan (*30*), occult virus were released from a binding site or from some nonsusceptible "carrier" cell, the virus might find a large population of susceptible cells in the thymus of an unshielded, irradiated host, and could initiate the sequence of intracellular events leading to lymphoma development. Conversely, the thymus of marrow-shielded animals, regenerating more quickly, might contain similarly undifferentiated cells for too brief an interval or in such small numbers that virus proliferation could not readily attain a self-sustaining equilibrium, and few lymphomas would result.

Although this hypothetical model explains certain hitherto puzzling phenomena, its experimental validation must unfortunately await the development of the sensitive virus-assay method referred to previously. The exquisitely refined potentialities of *in vitro* virus-culture systems should not deter us from continued exploitation of whole animal work, however, since the experimental alteration of host response may reveal important parameters of virus-induced neoplasia which are not apparent at the cellular level.

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Discussion

Dr. Lloyd Law (National Cancer Institute): I would like to congratulate Dr. Kaplan on this provocative paper. I have two comments pertaining to the abstract Dr. Kaplan submitted, which concern interpretations of our present knowledge in this field. He says in the first paragraph that in the past several years it has become well established that the lymphosarcomas and certain lymphatic leukemias which arise spontaneously in certain mouse strains, AK and C58, are due to a virus. I wonder if he really means that our present knowledge indicates this. As I understand it, the present knowledge reveals that certain agents or viruses, obtained from AK and C58 leukemias, are leukemogenic under certain circumstances.

Dr. Gross originated several concepts, one of which is "vertical transmission of the virus"; another, which he called "egg-borne" disease. As I understand, these are still concepts, but I did not know that these are well established at this time.

Also, Dr. Kaplan has confused the agent which is described by Rudali, Duplan, and Latarjet, if I understand the data correctly, in saying that the Gross agent not only induces leukemia but accelerates leukemia in the strain of origin. I think there is enough evidence to indicate that these are probably different agents. The agent which Rudali, Duplan, and Latarjet have described is sensitive to cold, and is not infective, for example, in C3H mice. They have shown that it is not transmitted to the next generation, and was obtained only from leukemic tissue and not from normal tissue of AK mice (*see* Latarjet, R.: Carcinogenesis. *In* Ciba Foundation Symposium, Boston, Little, Brown & Co., 1959, p. 274.) I mention these only because as a discussor I am supposed to try to stimulate the discussion.

At this point it would be well to give an idea of the biologic response of mice to X irradiation. Dr. Kaplan has talked about a special and unique case in which thymic lymphosarcomas result after certain optimal treatments with radiation: The irradiation has to be fractionated and whole-body, and there appears to be an extremely high threshold. I do not know what it is for C57BL mice, but for the CBA strain, as reported by Dr. Mole, the total dose is about 500 r. On the other hand, as discussed by Dr. Mole, the generalized lymphocytic neoplasms arise at much lower total doses and appear throughout the life of the animal, whereas thymic lymphomas appear chiefly within the 1st year. (*See* Mole, R. H.: Brit. M. Bull. 14: 174, 1958.)

There is also a difference in response, as shown by Furth and Upton, in the induction of myeloid leukemias in RF mice, in which 150 r, which does not induce thymic lymphomas, is effective in inducing myeloid leukemia. Thymectomy is not effective, and partial shielding, though slightly effective, does not completely eliminate myeloid leukemia. Also, if C57BL mice are thymectomized, there is a different story: Practically 50 percent are reticulum-cell sarcomas, which develop after 1 year of age, and rather late in life after irradiation.

I would ask Dr. Kaplan and Dr. Gross also, since Dr. Gross has reported on a similar type of work in the C3H/Bi substrain of mice, whether they really believe that there are adequate controls at the present time. For example, Dr. Gross reported an 11 percent incidence of leukemia in C3H/Bi mice after the introduction of extracts from X-ray-induced lymphomas. He found that serial passage was very effective in decreasing the latent period and increasing the incidence. Similarly, Dr. Kaplan showed a 20 percent decrease in the first passage material and indicated that as high as 70 percent was attained after serial passage. The control, it seems to me, should have been done in an analogous manner, *i.e.*, serial passage of material from a similar untreated source during the same interval.

Another question I think should be raised—and I am doing this for discussion—is whether or not there is any question of contamination, in these laboratories, by the agent which is obtained from AK mice. Dr. Kaplan has said that he has AK controls, and it would be interesting to have some idea of how many AK leukemias were tested in C57BL recipients. Dr. Gross has shown that not all of them reveal infective material.

Dr. Kaplan suggested that he is observing an activation of a virus, and I wonder if there are enough data to rule out the possibility of changes in the sensitivity of the host cells. For example, if one irradiates adult mice prior to the introduction of polyoma virus or introduces the virus and then irradiates, they become sensitive to the virus and tumors are induced. I do not know of any evidence which indicates that there is any activation of the polyoma virus. But this is a difficult problem, and some of these answers will be hard to obtain. As Dr. Kaplan suggested, there is no readily available and rapid assay method for leukemia.

Dr. H. S. Kaplan: I am grateful to Dr. Law for his discussion because it affords an opportunity to make a few additional remarks. As far as I am concerned, the evidence indicates that there is a virus that can be extracted from AK leukemia tissue, which will produce an identical neoplasm in the refractory C3H strain.

We have used some of Dr. Gross's passage A agent and have obtained tumor yields in C3H mice in excess of 60 percent in about the same interval that he has indicated in his published reports. Dr. Law said that I confused the acceleration phenomenon of Rudali, Duplan, and Latarjet with Dr. Gross's agent. That is not the case. We have taken the passage A agent, obtained from Dr. Gross, and put it into newborn AK mice, and obtained an acceleration phenomenon with over a 60 percent incidence by about 4 months of age, at which time none of the control AK mice have contracted leukemia.

Dr. Law's comments regarding the radiation threshold and Dr. Mole's work on thymic tumors versus generalized tumors seemed to me to be somewhat beside the point. It is perfectly true that I did not talk about myeloid leukemias, but I also did not talk about mammary carcinoma, salivary-gland tumors, carcinoma of the skin, or other kinds of cancer. This is one particular system. It is the only one that has been characterized with regard to the phenomenon that is under discussion, and I did not mean, even by exclusion, to indicate that the other areas are not worthy of study. But in the time available the subject has to be limited. I think Dr. Mole's data are essentially irrelevant to the present discussion.

As for reticulum-cell sarcoma, we have recently completed an experiment in which we used nonirradiated C57BL mice, thymectomized nonirradiated C57BL mice, and thymectomized irradiated C57BL mice, and the final incidence of this sarcoma was essentially identical in all three groups. There is no indication that a relatively large dose of radiation had any influence whatever on the development of this disease, and I do not believe that there is any basis at the present time for assuming that this is a radiation-induced reticular neoplasm. I think it is a spontaneous late tumor of these mice, and radiation has nothing to do with it.

As far as adequate controls are concerned, I mentioned some that we have at the present time. We have not done serial passage of control material, but we consistently get an incidence of 15 to 20 percent with either fresh material or with cell-free filtrates from an originally radiation-induced tumor which has been carried for a good many years in our laboratory, and we consistently get an incidence of zero with any of the control materials we have used, including cell-free filtrates from normal thymus. I suppose serial passage would be a good idea, but it has not been done.

Through the kindness of Dr. Huebner, we have recently tested some of our animals for polyoma virus and we do not seem to have the polyoma virus in our colony. Whether we have contaminated our C57 with the AK virus, I do not know.

About "activation," I have always used this word in quotes deliberately to emphasize that I do not know what activation means. The virus is presumably in the tissues all the time, yet radiation is necessary to bring out the tumor. I do not know whether I am talking about a change in host sensitivity or a change in the virus, but I would be inclined to think it a change in host sensitivity or host response rather than a radiation-induced change in the virus.

Dr. A. C. Upton (Oak Ridge National Laboratory): Dr. Kaplan has presented an extremely interesting and provocative observation. I was tempted, on reading the abstract, to view the priming effect, so to speak, as an example of tumor initiation and the effects of subsequent irradiation without shielding as promotion.

In an effort to ascertain whether in our system, the RF mouse, radiation might act as a promoting agent as well as an initiating agent, we have been comparing the susceptibility of irradiated adults and nonirradiated adults to the leukemogenic action of filtrates from lymphomatous tissue and tissue of animals with myeloid leukemia. We are getting some indications that the irradiated adult does show an enhanced yield of granulocytic leukemia and lymphoma when injected with filtrates from leukemic donors, which suggests that radiation does change the responsiveness of the host. [Carcinogenesis, Mechanisms of Action. *In* Ciba Foundation Symposium. (Wolstenholme, G. E. W., and O'Connor, C. M., eds.). London, J. & A. Churchill, Ltd., 1959, pp. 249–268.]

It is distressing to us to find inconsistencies in our results from one experiment to the next. I am wondering what this may denote in terms of activity of passage materials or host sensitivity. Dr. Donald F. Parsons and I have been attempting to screen our passage materials with the electron microscope, hoping possibly to find a correlation between particle content and subsequent leukemogenic activity on inoculation.

In this connection, it is of interest to inquire whether there is any information as yet about the mechanism of the gradual enhancement of tumor-inducing potency of passage materials reported by Dr. Moloney and by Dr. Gross. Are these merely quantitative changes, *i.e.*, increase in titer, or qualitative changes in the agent, *i.e.*, selection or possibly mutation toward increased tumor-producing virulence? These are old questions in virology; I am wondering if we have relevant information on leukemia viruses.

Finally, to return to host resistance, our use of the irradiated adult RF recipient was based on the fact that the neonatal RF recipient is refractory to the induction of granulocytic leukemia by radiation (Cancer Res. 18: 842–848, 1958). This raises another question. Is this really the result of host variables or are we dealing with epidemiologic factors? We have to consider the probability of contact between the host and the virus which is encountered in the environment. Might these be an influence of passive immunologic resistance acquired through maternal antibodies? The epidemiology of the tumor-inducing material is, thus, another problem about which little has been said but which is certain to plague us as we try to reproduce results from one time to the next and with animals of different colonies and different ages.

Dr. L. Gross (Veterans Administration Hospital, Bronx, N.Y.): Dr. Law mentioned that there is a question whether the virus etiology of AK or C58 leukemia is an established fact. The fact that filtrates, prepared from AK or C58 leukemic donors and injected into newborn C3H mice, reproduced faithfully the same disease is now confirmed in many laboratories. Dr. Law felt that this would only indicate that a leukemic agent from AK leukemic tissues could be extracted. In certain instances too much criticism may create confusion, because under such conditions we could not speak of Shope rabbit papilloma virus or of Rous sarcoma virus. Everybody knows that a filterable agent from such tumors as the Shope rabbit papilloma, the Rous chicken sarcoma, or mouse mammary carcinoma can be extracted. Upon inoculation into susceptible animals, these agents reproduce the respective neoplasms. Accordingly, it is assumed that each of these three different viruses is the causative agent of the neoplasm from which it can be consistently recovered and which it reproduces after inoculation into a susceptible host. The same applies to mouse leukemia.

I am satisfied that AK leukemia in mice of the AK and C58 strains is caused by a virus and I assume that such a virus is transmitted from one generation to another because from normal AK or C58 embryos a leukemic agent could be recovered.

Many new problems are facing us, however, which are difficult to solve. We have carried out experiments similar to Dr. Kaplan's, as far as radiation-induced leukemia is concerned, with C3H mice which in our laboratory do not develop spontaneous leukemia except in rare instances. We induced some 50 to 60 percent leukemias in C3H mice by fractionated total-body X irradiation. This leukemia could then be passed by filtrates. At first the incidence was low, but, by selecting an active filtrate and passing it through newborn mice, we eventually obtained an active agent designated "passage X" which induces an incidence of up to 80 percent of lymphatic leukemia within only 3 to 4 months after inoculation into newborn C3H mice.

Is passage X different from passage A which we previously isolated from spontaneous AK leukemia? What is the relation between passage X, passage A, or any AK leukemic filtrate and Dr. Moloney's leukemia? I am not sure that the routine morphological methods will give a prompt answer to these questions. For instance, the passage A virus in our current experiments can be inactivated after heating at only 50° C. for ½ hour. Is this also true for the other leukemic viruses? Thymectomy will make C3H mice completely resistant to the passage of strain A virus. Thymectomy, however, will not affect the susceptibility of mice to the Friend virus, and apparently will have little effect on susceptibility to the Moloney virus. These may be some of the tools useful in attempts to differentiate these viruses.

The main problem we are facing now is: Do we have a single leukemogenic virus in our mice, or do we have a family of leukemogenic viruses?

Dr. E. A. McCullough (University of Toronto): I would like to ask Dr. Law a question. Some years ago he was coauthor of a paper in which he reported the injection of bone marrow into a cross between strains C3H and AK in which, if parental C3H marrow was used, the incidence in the F_1 hybrid was reduced, and, if AK marrow was used, the incidence in the F_1 hybrid was markedly increased. [Lorenz, E., Law, L. W., and Congdon, C. C.: The role of bone marrow and spleen in induced and spontaneous leukaemia. Leukaemia Research. *In* Ciba Foundation Symposium (Wolstenholme, G. E. W., and Cameron, C. M., eds.). London, 1954, pp. 189–195.] I wonder if now, in view of Dr. Kaplan's results and in view of the importance of virus in leukemogenesis, he feels that in this situation the bone marrow was acting as an antiviral agent. For the sake of argument, it might be assumed that the virus comes from the AK mice. Does this make the action of bone marrow a little more general than Dr. Law would allow Dr. Kaplan to have it?

Dr. Law: I do not quite understand your question, but I think that both Dr. Kaplan and Dr. Gross missed the point of my first comment. I said that, in my opinion, the present knowledge in this problem concerning AK and C58 leukemias is that there is obtainable from AK mice an agent which is leukemogenic. But it seems to me, when you say that this agent is responsible for the spontaneous leukemias in AK and C58 strains, it is still a concept, because there is no proof as far as I know. The explanation is in the form of a concept and must be proved. Why have strains AK and C58 for 20 or 30 years, generation after generation, shown high rates of lymphocytic neoplasms? What is the nature of the transfer of virus?

There is no doubt that these materials are leukemogenic in the recipient mice into which they are introduced, and I am not arguing that point at all. When they are considered responsible for the spontaneous leukemia of these strains, this is another question. I do not know that anybody has an answer as to how the bone marrow acts in the prevention of X-ray-induced leukemia.

Dr. Kaplan: I do not believe that we will get much further with this argument. It becomes a question of semantics whether something is an established fact if any one individual in the world does not believe it.

I would say that this is a problem relating to the philosophical nature of evidence on causality. I feel that Dr. Gross's agent is extracted from leukemia, that it will produce morphologically identical leukemia in other strains, and that it will accelerate the appearance of morphologically identical leukemia in the strain in which it was first encountered.

I believe that we will get further by advancing with the problem in the laboratory. Once we succeed in establishing *in vitro* systems, in which these extraordinarily bashful leukemia viruses can be studied more directly, we can clarify many currently troublesome problems. If we use up too much of our energy at the present time in arguments of this sort, I think we will hold back progress.

The radiation-induced lymphoma is interesting to compare with the spontaneous lymphoma. The spontaneous AK system apparently does not require any radiation, and one wonders why not. I think it would be interesting to go back to the AK tissues and see if there are any indications about this. On one point I will definitely agree with Dr. Law: We are still in the dark as to the pathogenesis of leukemia in AK mice even if it is conceded that they have a virus. It takes them months to get the disease, and we have very little understanding of what finally triggers this response.

There is a great need to return to the AK system and study it further. But, in the irradiated system with which we have worked, it appears that radiation is a leukemogen, whereas many other agents are not leukemogens. Radiation, unlike all the other agents we have studied, does two things, and both of these must apparently occur in order to produce leukemia: (1) damage to the thymus; I would guess that in the course of that injury virus is released from some thymus cells in which it has been confined; (2) damage to the bone marrow, which in turn interferes with the recovery of the thymus, and as I have shown, the nature of that interference seems to have something to do with a maturation arrest.

Cultivation of the Mouse Mammary Carcinoma Virus [1,2]

ETIENNE Y. LASFARGUES, MARGARET R. MURRAY, *and* DAN H. MOORE, *Department of Microbiology and Surgery, Columbia University, and the Rockefeller Institute, New York, New York*

THE description in 1948 by Porter and Thompson (*1*) of particulate bodies associated with neoplastic mammary cells observed in electron micrographs furnished a strong argument in favor of the viral nature of the milk factor. However, two important links were still missing: (1) No correlation could be established between the particles observed and the carcinogenic activity of the milk, and (2) there was no satisfactory evidence that the agent could be isolated from the tumors and propagated independently in tissue culture.

Two years ago, Moore *et al.* (*2*) were able to show by physical procedures that the particulate bodies seen with the electron microscope are endowed with infectivity, and thus succeeded in identifying them with the milk agent. At the same time strong evidence was obtained that the mammary-tumor agent (MTA) multiplies in tissue culture (*3*).

Earlier attempts by Taylor and coworkers (*4–6*) to maintain the milk agent in embryonated eggs had given controversial results. However, Bittner, Evans, and Green (*7*) recovered an active agent from the yolk 12 days after inoculating eggs with a cell-free tumor filtrate. Also, by injecting mice with an agent maintained through several passages on cultures of chicken fibroblasts, Pikovski (*8*) increased the tumor incidence in a colony in which it was usually low. More recently we observed that the particulate bodies released by the neoplastic cells could increase considerably in number simply after a few hours of cultivation in roller tubes (*9*). These were definite indications that the MTA can be maintained, possibly multiplied, outside its natural host with conventional techniques of virus production.

POSSIBILITIES FOR CULTIVATION

Several facts were known concerning the milk agent: For instance, we knew that it can pass through Seitz and Berkefeld filters (*10*) and is

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[1] Presented at a Symposium on Phenomena of the Tumor Virus, New York, N.Y., March 25 and 26, 1960.

[2] This investigation was supported by research grant C-2520 from the National Cancer Institute, National Institutes of Health, Public Health Service.

transmissible through milk. When injected into susceptible mice, it induces the formation of mammary tumors in 10 to 15 months. Attempts to shorten this latent period have so far been unsuccessful. Since none of the current serological or immunochemical methods are accurate enough to detect the MTA, bioassays remain the only available test. Through bioassays, it has been established that the cancerogenic power of milk, or *infectivity,* is heat labile and is completely destroyed in a little more than *1 hour* at 37° C. (*11*). This could be a handicap for its cultivation; on the other hand, that it has been found stable in a range of hydrogen ion concentration varying from *p*H 5. to 10.2 (*12*) is an asset, because the *p*H of tissue-culture media is usually adjusted to neutrality. Two other factors could be of consequence for successful cultivation: (*a*) the source of the agent, (*b*) the choice of a tissue substrate.

The milk from RIII mice has been found a potent source material. Injected into susceptible mice, it can induce tumors even after dilutions up to 10^{-10} or 10^{-12}. The test mice used for all our bioassays are from our Columbia C57BL strain sensitive enough to respond to such high dilutions, yet completely agent-free, since not a single spontaneous tumor has been recorded in the colony in the past 12 years.

Such mice were indicated to provide the tissue substrate of the cultures, and since the milk agent has a special affinity for the mammary epithelium, the mammary epithelium had to be grown. For many years, the growth of this tissue met with considerable difficulties. In 1950, Hardy (*13*) demonstrated the possibility of obtaining differentiation in organ cultures of rudimentary mammary systems from the abdominal skin of mouse embryos. We adopted this method in our first experiments. Later on, however, the enzymatic treatment of adult mammary glands enabled us to grow sheets of mammary epithelium in conventional roller tubes (*14*).

ORGAN CULTURES

When the skin from the abdominal wall of mouse embryos taken at mid-pregnancy is explanted on a nutritive gel as described by Hardy (fig. 1), primary and secondary mammary ducts differentiate from the skin epidermis. This type of culture is necessarily complex, since a variety of tissues are growing in an organized fashion. The mammary ducts expand in the underlying connective and adipose tissues where they appear, after histological section, as tubes limited by 2 or 3 rows of cuboidal cells. Besides the mammary epithelium, which is our main concern, and its loose connective environment, the hair follicles, the germinal layer and the keratinized cells of the skin still represent an important part of the culture. The ratio of mammary epithelium thus obtained is therefore extremely small. The maintenance period of such cultures is also relatively short since, after 10 to 12 days of explantation, foci of necrosis develop rapidly. In our first two attempts this procedure was followed:

Fragments of embryonic abdominal skin from agent-free C57BL mice are explanted on a nutritive gel containing defatted milk from the RIII strain in the final dilution of 10^{-3}. After 10 days, the fragments are ground in their medium and centrifuged. The supernatant is in part inoculated intraperitoneally into C57BL baby mice while another part is diluted a thousand times in the medium of a new set of cultures. In this manner "passages" of the primary inoculum are made with 1000-fold dilutions for each 10-day period of maintenance *in vitro*. The results of the bioassays which became apparent 12 months later are summarized in table 1.

TABLE 1.—Tumor induction with organ-culture medium

	Concentrations of MTA* inoculums	Number of mice inoculated†	Number of induced tumors	Time of cultivation (*days*)	Time of appearance of first tumor (*months*)
Controls, acellular medium	MTA 10^{-3}	62	37		10
	MTA 10^{-12}	40	2		11
	IMTA‡ 10^{-3}	60	0		
Culture extracts (relative dilution of primary inoculum)	CMTA§ 10^{-3}	62	5	4	14
	CMTA 10^{-6}	63	2	14	13
	CMTA 10^{-9}	62	8	24	10
	CMTA 10^{-12}	64	10	35	13

*MTA: mammary-tumor agent (RIII milk).
†C57BL strain, no spontaneous tumor in 12 years; intraperitoneal inoculation, 0.1 ml.
‡IMTA: incubated mammary-tumor agent (4 days at 37° C.).
§CMTA: cultivated mammary-tumor agent.

One can see that, while a dilution to 10^{-3} of fresh RIII milk induces tumors in 37 of the inoculated mice, only 2 appear after a simple dilution to 10^{-12}. Furthermore, the same milk loses all its carcinogenic activity after 4 days of incubation at 37° C. This we could expect, since other authors have reported a loss of activity after 1 hour at 37° C. Nevertheless the milk agent is retained in the presence of living tissues, as shown by the induction of 5 tumors at the end of the first passage. This number climbed to 8 on the third passage and to 10 on the fourth. By then the primary inoculum had been diluted to 10^{-12} and kept at the incubation temperature of 37° C. for 35 days. A comparison with the above acellular controls suggests a definite multiplication of the agent in the cultures.

ROLLER-TUBE CULTURES

In the meantime, techniques for the culture of pure normal mammary epithelium from adult mice were developed (*14*). A more or less prolonged digestion with collagenases separates the epithelium from the adipose tissue in which it is normally embedded. In prelactating mammary glands, an extremely well-developed epithelial system replaces almost completely the connective tissue usually abundant in a resting organ. Explantation of this material gives large monolayered sheets of thin epithelial cells in pure colonies (fig. 2).

With a procedure identical to the one devised with organ cultures, C57BL mammary epithelium grown in roller tubes receives the agent in the form of RIII milk diluted to 10^{-3} in the culture medium. Transfers are then made routinely once a month, in 1000-fold dilutions over a period of 1 year. The results of systematic bioassays on groups of 20 mice (table 2) show that only 2 tumors are induced after 1 month of cultivation, 2 after the second, and 1 after the third. It therefore appears that, though the milk agent can be maintained for at least 3 months in this type of culture, it does not multiply and is eventually eliminated.

Table 2.—Tumor induction with roller-tube culture extracts*

Dilutions of primary inoculum	Months of cultivation	RT+ MTA†	Controls, RT
10^{-3}	1	2	0
10^{-6}	2	2	0
10^{-9}	3	1	0
10^{-12}	4	0	0
10^{-15}	5	0	0
10^{-18}	6	0	0
10^{-21}	7	0	0
10^{-24}	8	0	0
10^{-27}	9	0	0
10^{-30}	10	0	0
10^{-33}	11	0	0
10^{-36}	12	0	0

*Twenty mice were inoculated each time both with the experimental and control media.

†RT: roller-tube extract; MTA: mammary-tumor agent.

Two possible alternatives can be considered to explain such results: (1) Instead of from embryonic tissues, the roller-tube cultures are derived from adult mammary glands in the prelactation stage. It has been shown by Andervont (*15*) and Dmochowski (*16*) that a natural resistance to the agent develops in older mice. This is particularly true of the C57BL mice not genetically inclined to transmit the milk factor. (2) The second alternative is the difference in complexity between organotypic and conventional homogeneous cultures. Could it be possible that the multiplication of the factor is dependent on specific metabolites supplied by the surrounding tissues?

Embryonic Tissues

The first consideration is easy to test. Since the mammary epithelium differentiates in organ cultures from the epidermis, whole skin from 10- to 12-day embryos should provide a good tissue substrate for virus multiplication. Proceeding in the same manner as with the adult mammary epithelium, we made cultures of embryonic skin and inoculated them with RIII milk diluted to 10^{-3} in the medium. The infectivity was tested by bioassays through 4 successive passages and 1000-fold dilutions. Three tumors could be induced with the culture medium after

3 days of incubation and only one after the first passage (21 days). From a total of 130 experimental mice it is evident that the embryonic quality of the tissue is not alone sufficient to insure the proliferation of the agent.

Glandular Stroma

In regard to the second alternative, indications that the surrounding stroma is physiologically connected with the mammary epithelium have already been obtained (*17*). When, after a prolonged dissociation by collagenase, a mammary gland yields cultures of pure epithelium, one is surprised to find no major vacuole or lipidic inclusion develop in the clear cytoplasm of these cells. Even though the mammary glands are, in most cases, in the prelactating stage, lipides are not formed by the epithelium. If the mammary epithelium is explanted while in full secretion, there is an abundance of lipides at the start, but this very abundance wears off as the culture is prolonged. It all appears as if the lipides secreted were of exogenous origin. This becomes more evident when the mammary glands of a mouse taken at mid-pregnancy are explanted, one after complete dissociation by collagenases, which gives pure mammary epithelial cultures, the other after a short enzymatic action, which retains a great part of the adipose tissue of the gland. Numerous lipidic inclusions in the mammary epithelium are observed only in the presence of the adipose tissue; none are seen in cultures of pure epithelium. The elaboration of milk lipoproteins by the mammary epithelium therefore depends on the surrounding tissues of the glandular stroma.

As shown by the results obtained in organ cultures, the multiplication of the milk agent may also depend on the environment. Roller-tube cultures from prelactating mammary glands, partially dissociated with collagenase in order to retain part of the stroma, are then inoculated, as previously, by adding RIII milk in the standard dilution of 10^{-3} to the medium. After 3 weeks of maintenance with 2 changes of medium a week, the cultures are ground, centrifuged at low speed, and the supernatant injected into C57BL mice.

Ten of the 15 experimental mice developed a mammary tumor (table 3), the first one appearing less than 9 months after the bioassay. The per-

TABLE 3.—Influence of the glandular stroma on the maintenance of the milk agent *in vitro*

Inoculum	Number of mice inoculated	Number of tumors induced	Tumors (*percent*)
RIII milk 10^{-3}	15	8	53
IMTA*	15	0	0
Partially dissociated gland. Control culture 10^{-3}	15	0	0
Partially dissociated gland + MTA† 10^{-3}	15	10	67
Totally dissociated gland + MTA 10^{-3}	20	2	10

*IMTA = incubated mammary-tumor agent (3 days 37° C.).

†MTA = mammary-tumor agent.

centage of tumors induced is even higher than after the inoculation of fresh milk. In comparison, no tumor appeared in the animals injected with the extracts of control cultures which did not receive RIII milk. If one recalls that only two tumors had been induced in 20 mice by cultures of pure mammary epithelium treated in similar conditions, one must conclude that the glandular stroma is somewhat involved in the multiplication of the milk agent.

DISCUSSION AND CONCLUSIONS

Because of its far-reaching implications, this last experiment must be further confirmed. There is no doubt, however, that the milk agent can multiply independently in tissue culture, outside its natural host. In this respect, and also because of its structural appearance as revealed by the electron microscope, one has to consider the MTA as an authentic virus.

The mouse mammary virus has been found in the liver, lungs, kidneys, spleen, thymus, heart, blood, and seminal vesicles (*18*). It is significant that, despite this wide distribution, it induces tumors only in the mammary epithelium. It can therefore survive for long periods in various locations, but finds the conditions necessary for tumor formation only in one elective site, the mammary gland. Tissue-culture techniques indicate another selective characteristic that thus far has not been displayed by other kinds of viruses or expressed in definite terms: The milk agent will develop in the mammary epithelium only if this epithelium is "conditioned" by the proper environment, *i.e.*, by the glandular stroma, possibly by a particular state of this stroma.

We know that the mammary gland is an organ subject to periodicity and that its functional activity, under critical hormonal control, is accompanied by important structural and biochemical modifications (*19*). Under the influence of the hypophyseal hormones, for instance, the epithelial parenchyma varies tremendously in volume from the resting stage to full lactation, then through involution back to the resting stage. The ovarian hormones, on the other hand, are powerful modifiers of the stroma but, at the same time, spreading factors such as hyaluronidases appear, which are of fundamental importance in the prelactating period. Mammotropin also initiates an increased fat elaboration in the adipose tissue and so probably influences the reactions of enzyme systems such as diastases, phosphatases, lipases, and dehydrogenases, which have been reported as normally present in this tissue (*20*). We have shown that secretion by the mammary epithelium is connected with the activity of the adipose tissue (*17*). If, as suggested by our last experiment, the multiplication of the MTA is influenced by similar factors, its synthesis in the epithelial cells may also depend on the presence of a specific metabolite supplied by the surrounding tissues.

Such a mechanism would explain some of the oddities of this virus. Because of the periodic function of the mammary gland, any specific metabolite may be available only at one definite stage of the glandular cycle. The synthesis of virus proteins cannot therefore be continuous, but strictly limited to this period. Before, or after, the virus just survives as it does in the other parts of the body, but in the mammary gland it will resume active multiplication whenever the conditions permit. This would be consistent with the fact that tumors appear generally late in the life of the mouse, usually after several pregnancies. It is also consistent with the heritable hormonal pattern that has been advanced to explain the differences in tumor incidence between susceptible and resistant strains (*21*). Variations in the normal secretory output of hormones involved in the mammary-gland control will evidently "condition" the tissues of this organ differently. In this respect, it is significant that the RIII strain of mice, which has a high incidence of spontaneous tumors, is a great milk producer, while the C57BL strain, resistant to the milk agent, gives in similar conditions a very small yield. It is no less significant that hyperplastic nodules with a typical lobulo-alveolar structure develop in virgin RIII mice and not in the C57BL. Pitelka *et al.* have shown these hyperplastic nodules to be a site of virus multiplication (*22*), but Nandi (*23*) demonstrated that lobulo-alveolar development is possible only under the combined action of ovarian hormones, deoxycorticosterone acetate, and either prolactin or growth hormone. These hormones, probably more readily available in the young RIII than in the C57BL strain, appear as factors preparing the ground for virus multiplication rather than as agents directly involved in mammary carcinogenesis.

Another fact in support of an exogenous complement necessary for the epithelial cells to synthesize new virus proteins is given by cultures of mouse mammary tumors. In the 48 hours after the primary explantation of neoplastic cells, a large amount of viral particles is produced, which can be demonstrated by electron microscopy in the culture medium itself. Since we considered this method as a possible way of producing large amounts of virus, we checked systematically the medium of tumor tissues maintained in continuous culture. It was observed that the viral particles, so abundant in the first days of cultivation, were thinning away from the 10th day down to the 15th or 20th day, after which particles were seldom seen. Since the cells themselves remain in excellent morphologic condition and the composition of the medium is constant, it seems that the loss of the viral particles is caused by default of a necessary component explanted with the primary tissue but exhausted after a few days of life *in vitro*.

Several points remain to be clarified to have a precise picture of the mechanisms involved in mouse mammary carcinogenesis. The results we have presented, however, offer an opportunity for a more logical experimental approach. If we succeed in obtaining a continuous production of the mammary-tumor virus *in vitro*, several of these remaining problems will eventually be solved.

To summarize: Evidence of multiplication of the milk agent has been obtained in organotypic cultures of embryonic mouse skin in which mammary rudiments differentiated from the epidermis. However, conventional roller-tube cultures of embryonic skin epithelium failed to maintain the virus for any length of time. Maintenance without multiplication was achieved in cultures of pure mammary epithelium from the adult mouse. On the other hand, there are definite indications that the virus will grow on adult mammary epithelium, if a part of the adipose tissue from the glandular stroma is retained in the culture. In the light of this last observation, it is suggested that synthesis of virus proteins by the mammary epithelium is mediated by specific metabolites supplied by the surrounding tissues.

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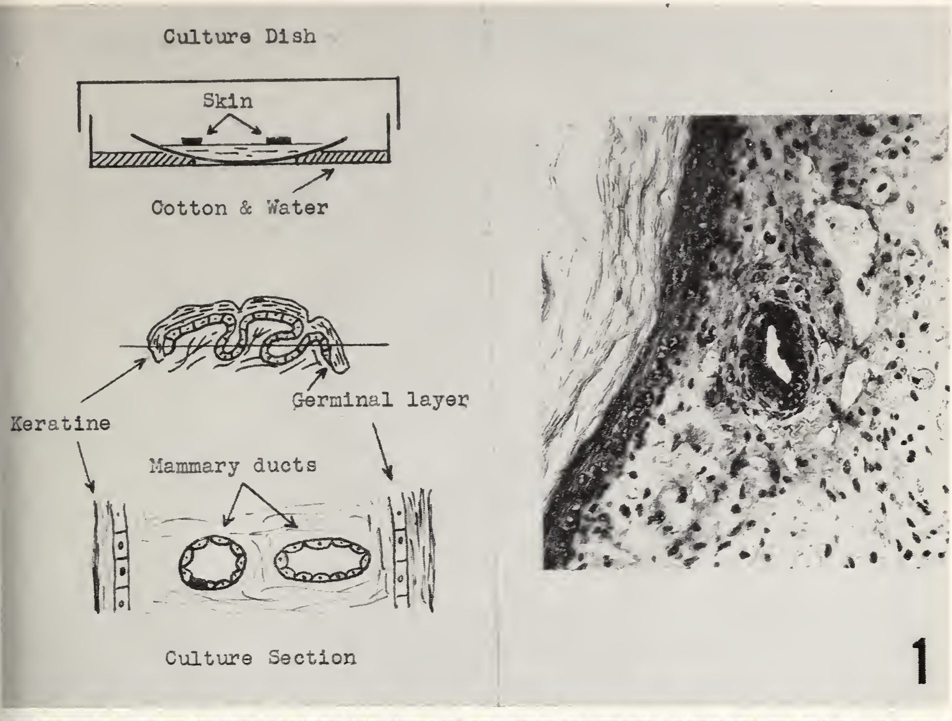
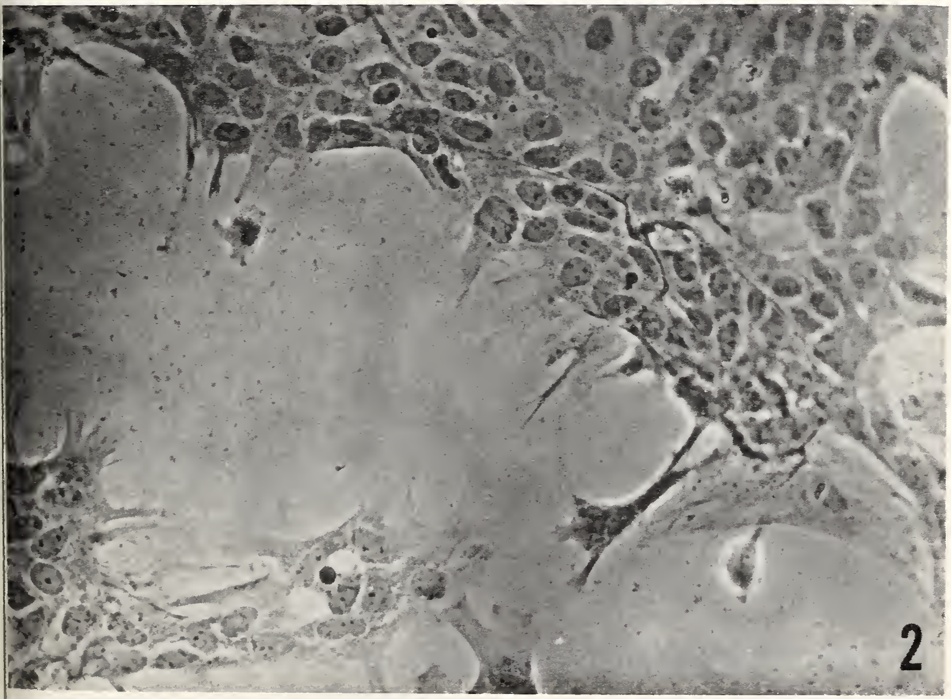
PLATE 24

FIGURE 1.—Hardy's technique of organ culture. Fragments of embryonic skin from 12-day mouse embryos are placed on the nutritive gel with the germinal layer down. Mammary tubes differentiate in underlying connective and adipose tissues.

FIGURE 2.—Expanding mouse mammary epithelium; 7-day culture from adult C57BL mammary epithelium explanted at mid-pregnancy. × 240


Culture Dish
Skin
Cotton & Water
Keratine
Germinal layer
Mammary ducts
Culture Section

1



2

Discussion

Dr. Dan Moore (Rockefeller Institute): I suppose we should follow the previous procedure and be provocative in our statements. Therefore, I will make a few statements that I am sure will be provocative.

Our present work with the milk agent is based on the following, which might be called a sort of creed: We believe that the Bittner agent is really a virus and that it is carried in a large number of strains of mice. Actually, we believe that most mice carry this agent, and there are really few that do not. There are about nine strains, now reported in the literature, in which the milk agent has been found, even some that are not supposed to have it.

Also, we believe that a primary approach to this kind of investigation should include tissue culture. Dr. Lasfargues has just demonstrated what can be learned with this technique.

We believe that we are able to recognize this agent morphologically wherever we find it in tissue. I am not absolutely sure of this, however, because these tumor viruses can look a whole lot alike, and unless one takes some care in measuring the parameters, it is possible to mistake the virus.

Furthermore, we believe that we are able to trace the development of this agent at the cell membrane, that the entire assembly takes place at the cell membrane, and that the particles are liberated in acinar or intercellular spaces. The cell membrane thickens, protrudes to form a hemisphere, then a horseshoe-shaped spheroid which finally pinches off. The newly formed thick-shelled spheres are of remarkably constant diameter, *ca.* 95 mμ. Particles slightly removed from the cell membrane contain a dense nucleoid within a membranous sac, which indicates a complete rearrangement of dense material soon after the particle is formed.

This process is clearly indicated in the mammary carcinomas of DBA mice kindly given to us by Dr. Anna Goldfeder of the Delafield Hospital: The production of milk agent is greater in these DBA tumors than in any other strain so far studied. The appearance of particles is the same, however, as has been described for other strains including the cultured explants from RIII tumors.

The ubiquity of these milk-agent particles in mammary carcinomas is demonstrated by their occurrence in Heston's (National Institutes of Health) C3Hf mice, a so-called agent-free strain kindly supplied by Dr. B. D. Pullinger of Glasgow. The concentration of particles in these tumors, however, appeared to be relatively very low. This low concentration may account for the difficulty in transmitting the disease from this strain.

Dr. Mühlbock: Everyone concerned with the milk agent has followed with great interest the work done by the Columbia group. I think everyone agrees that their approach to the problem is sound. It would appear from Dr. Lasfargues's report that one of the major difficulties in this field, *i.e.*, the test for the milk agent, has been overcome. Otherwise, one must rely on the biologic test, which takes a long time and is a handicap to be overcome if we want to proceed more rapidly in the future.

Maybe the approach Dr. Lasfargues has found in tissue culture will help in this respect. His conclusions from the work in tissue culture agree with what we see with purely biologic investigations. The role of the connective tissue is certainly very important, and I am glad he stressed this point.

It was gratifying that he emphasized the role of hormones in their influence on the conditions necessary for the modification or multiplication of the milk agent. He indicated, himself, that the evidence for this is not very convincing, and I wonder if he would comment in this respect. First, does the agent multiply in the mammary gland in the prelactation stage? After lactation the mammary gland practically disappears by involution, and I would like to ask him what happens to the virus, then, which has multiplied. As another point, we know that the milk agent can multiply in male animals in which there is hardly any mammary gland at all, and we are sure that the mammary-tumor agent can be transferred very easily with the sperm in certain strains of mice.

Dr. Sykes (M. D. Anderson Hospital): Contrary to the findings of Dr. Lasfargues, we in Houston have found that the cells of mammary tumors from agent-free, or supposedly agent-free, mice grow out as beautiful epithelial sheets. The cells are very regular, and no abnormalities are found in them, when they are viewed by phase microscopy.

In contrast, the cells growing out from tumors of mouse strains carrying the virus are bizarre in shape. They are quite unlike normal epithelial cells, tend to be multinucleate, to form giant cells, and to be highly vacuolated, even after 2 years in culture.

The mice providing the spontaneous tumors were the C3H/He, C3H/Bi and C57BL strains. The agent-carrying strains have been the equivalent of these three, and, in addition, the Strong A strain.

Just to confound the issue a little more, one of the spontaneous tumors from a C3H mouse carrying the agent was passed 19 times in the chick-embryo yolk sac and, thereafter, 69 times in tissue culture over a period of 2 years. The cells of this culture have already shown the presence in electron micrographs of characteristic virus particles.

Dr. Alice Moore: (Sloan-Kettering Institute): What I have to say is not related to the milk agent but does point out some of the difficulties of growing these tumor viruses in tissue culture. Dr. Friend described her agent about 4 years ago, and, ever since then, we have been attempting to grow the virus in tissue culture.

A couple of years ago we reported preliminary results indicating that the virus grew in a rather desultory fashion. Since then we have refrained from further publication in the hope that we could better the situation, but this has not been the case.

The results obtained with cultures of mouse embryo, infected either with filtrates or with cells from the mice with leukemia, showed that virus was in the culture supernatant fluid during the 1st month and caused leukemia in 4 of 10 mice inoculated. In the 2d month, no virus was demonstrable, but in the 3d and 4th months, it began to reappear. By the 6th month, there was a little more virus, which caused disease in 6 of 9 mice. Thereafter, it kept appearing and disappearing throughout a period of 21 months. We have had cultures over 2 years, and many of them still produce the virus, but in this rather relaxed fashion. It goes on and on, but we have never succeeded in getting any more virus.

This indicates to me that at least some of these tumor agents are going to be difficult to propagate in tissue culture and that we must either modify our present methods or develop new ones, such as Dr. Lasfargues has done.

Dr. Eddy (National Institutes of Health): An important point of Dr. Lasfargues's paper suggests a solution to Dr. Moore's problem. This is the observation that he was able to use the stroma cells, and I wonder if anything is being done along these lines, whether monolayer cells can be used with the addition, perhaps, of embryo extract or can the stroma cells be preserved in additive cultures? Would not that make possible propagation of the virus, in a fashion, much easier than that employed at present?

May I ask if cortisone or X rays do anything toward making the animals come down a little faster?

Dr. Sabin (Childrens Hospital Research Foundation, Cincinnati): The work presented has required so much courage and physical labor that I hope what I have to say will not be regarded as hypercritical but rather as a résumé of the important conclusions that have been reached.

It has been indicated that the milk tumor agent in culture medium without cells at 37° C. disappears within an hour. The fact, however, that the virus persists for months in cell culture with repeated fluid changes and still produces so many tumors certainly suggests that something has happened. But this may not necessarily mean that the stability of the virus in the culture containing cells is the same as that in the medium without cells.

I am led to this comment because several years ago when we tried to grow certain strains of polio virus in chick-embryo tissue cultures, we quickly found ourselves in a similar situation. The virus incubated in the medium without cells disappeared shortly, but the agent persisted for several weeks longer in the tubes containing the chick-embryo tissue. Furthermore, we would wash the cells and think that we had gotten rid of everything by dilution. After the cultures were in the roller drum for a time, there was the virus again. An explanation was related simply to a change in the *p*H produced by the metabolizing cells. When the *p*H was properly adjusted, the polio virus persisted at 37° C. without chick-embryo cells as well and as long as in cultures of the cells.

Thus it was learned that repeated washings did not remove virus from the cells as calculated by directly simple dilution, but that a certain amount remained adsorbed either through the plasma clot or on the cells, and it kept coming off.

Then it has been shown recently that certain amino acids increase the thermal stability of poliomyelitis virus. Thus it would seem necessary to control studies with respect to the possibility that either the *p*H produced by the metabolizing cells or certain amino acids liberated into the medium by the metabolizing cells (and perhaps they are even liberated by a stroma and not as much by epithelial cells) may in themselves contribute a more appropriate medium for stability of the virus. This might be tested by measuring the stability of the virus in the fluid from the control cultures, without the tumor agent, to see if there is a difference in the stability of the agent diluted in culture fluid from that of the agent diluted with fluid in which no cells had been grown. This should provide an informative control.

I was particularly impressed by the data that Dr. Alice Moore discussed. This business of a little virus coming along is very disheartening—now you see it; now you don't—and it goes on for 2 months, then 2 years. The stick-to-itiveness of these people calls for much admiration. These results definitely suggest virus synthesis at a level which is barely detectable. Now, again, I mention that the kind of pattern Dr. Moore described (and it may also apply to the data of Dr. Lasfargues) has been found with so-called classic viruses when either the *p*H of the medium or the temperature (there may be other factors) is just wrong. If a virus that multiplies optimally at 37° C. is cultured at a temperature only 2° higher, one may observe just this pattern. Or, with the reverse, if the temperature is decreased a few degrees, such a pattern may result. Now, it may be that the temperature of the mammary tissue is not like the 38° C. of the normal mouse. Perhaps it would be desirable to move the temperature down to 34° or to 30° C., and maybe, at those temperatures, more virus would be formed.

Dr. Sanford (National Institutes of Health): With Dr. Andervont (National Institutes of Health) we have been trying to learn whether the mammary-tumor agent can be maintained in long-term cultures of the mammary carcinomas that have arisen spontaneously in strain C3H mice. We have established about 10 long-term strains which have been examined for the agent for quite a long time. In most of these the agent was maintained in culture for at least 6 months. Three of the strains were continued for longer than 3 years. One of these was tested again after 5 years, both the original strain and single cell clones. These tests were exhaustively made by Dr. Andervont on the susceptible C3Hf mouse, and no agent could be detected.

When the cells were implanted into mice, they produced cystic tumors, *i.e.*, the clones, which were diagnosed by Dr. Dunn (National Institutes of Health) as anaplastic carcinoma. They were not differentiated carcinomas. The other two strains have been continued, and one of these reproduces the original tumor.

There is just slight evidence that a mammary-tumor agent is present. The data are almost too limited on the numbers of mammary tumors produced to be sure. These results, on carrying the mammary carcinoma in culture, resemble some of the data which Dr. Andervont and Dr. Dmochowski (M. D. Anderson Hospital) have collected on carrying some of these tumors for many passages *in vivo* in agent-free animals. In three of their studies, they have not been able to detect the agent after many transplants in the agent-free host, which again, of course, raises the question as to whether propagation of the virus is essential for continuation of the malignant properties of the cell.

I should like to ask Dr. Lasfargues whether he can strengthen up these points which Dr. Sabin raised with respect to the quantitative data on the propagation of the agent in the cultures. Now, he removes the used culture fluid, sediments the cells, and dilutes the supernatant fluid a 1000-fold to inoculate his mixed culture, as I understand his statements. I just wondered if there is a possibility that these supernatant fluids were not always cell-free. I wonder if he tried to filter the supernatant fluids to be absolutely sure, because it seems that, with this great dilution of material, he would have good evidence of propagation if he could be sure that these supernatant fluids did not contain cells.

An additional comment relates to the question of the role of the virus in the neoplastic change. We have isolated a single cell clone from normal parotid tissue of a strain C3H mouse and treated the cells in three separate experiments with the polyoma virus. We then implanted the cells, which were of C3H mouse origin, into hybrid mice and have obtained tumors in all three experiments with the treated cells but none from the controls. In order to be sure that these tumors were not induced in the hybrid host by virus, we have transplanted them and shown that they are specifically the C3H cells and not cells from the hybrid host. This is not closely related to your problem, but I did wonder if you had tried filtration of the supernatant fluids.

Dr. Dmochowski (M. D. Anderson Hospital): With respect to Dr. Dan Moore's statement earlier: Although it may be to him, and even to Dr. Bittner, a creed that the mammary agent is a virus, to some of us, at least, it is not a matter of creed but of scientific fact that the agent is a virus.

To another point which he made, I agree wholeheartedly that the mammary virus is probably more widely spread than we have realized. In Amsterdam in 1956, I brought up a similar point, and my English colleagues still criticize me strongly for expressing the idea of my chief, Dr. W. E. Gye, of a universal virus. It is not a matter of a universal virus, but it is a fact that the milk virus may be spread in all mouse strains. Some of it may not be present in some and only in small quantities in others, as may be exemplified by findings with the well-known C3Hf strain of Heston. On electron microscopic examination of tumor from several of these mice, we failed to find virus particles, while in others, we found them in small numbers. Biologic tests of some of these failed to show tumor-inducing activity, while others showing few particles by electron microscopy, exhibited very low tumor-inducing activity, about 5 percent. Concentrates of extracts from those tumors that have shown virus particles, when injected into suitable susceptible mice, induced up to 40 percent of tumors.

This is a matter which must be considered with tissue culture studies as well as those in animals. This would be of much interest because of the possible influence of culture medium and temperature on the increase of virus particles demonstrable morphologically and biologically.

Dr. Koprowski (Wistar Institute): Will Dr. Lasfargues and Dr. Dan Moore reply to the comments and statements made by the discussants?

Dr. Lasfargues: It is good to know that we are in agreement with Dr. Mühlbock concerning the influence of hormones on mouse mammary carcinogenesis. Dr. Mühlbock asked what the milk agent is doing while the mammary gland is in the stage of involution. The milk agent, of course, is present all the time. Baby mice of the C57 strain, for example, initially completely agent-free, become infected by the milk from the mother. The virus particles taken up through the digestive tract are distributed to various organs, including the mammary glands, though not necessarily in the epithelium.

At the time of pregnancy there is a release of hormones that modify the stroma and stimulate the activity of enzyme systems, which modify the properties of the cells and permit penetration of the epithelial cells by the virus.

The virus will multiply there during the time of pregnancy when the mammary epithelium is growing rapidly, and, at the time of lactation, will be released into the milk. In the period of involution many of the cells producing the virus will die, and the virus in them will be lost through the milk. The remaining cells, not being in the proper condition to synthesize the virus, will be in a state of latent infection, and it may not be possible to find any viral particles.

Thus it seems clear that the hormones acting on the epithelium and also on the stroma induce the formation of enzyme systems which are necessary for the synthesis of viral proteins. At all times other than during pregnancy, the virus is latent and is practically not to be seen.

Dr. Eddy asks if it would be possible to condition the tissue culture of one type of cell in roller tubes so that it would be able to support virus synthesis. It is always possible to combine the two types of cells, and it is regarded as a necessary combination. More and more workers have found that a combination of tissues is necessary for the maintenance and multiplication of some viruses. Dr. Algard, for example, in a study of the influence of hormones on hamster kidney tumors, found that the cancer could be induced only if he had a combination of several tissues. He could not obtain the expected reaction if he had cultures of pure cells. But perhaps it would be possible to condition cultures of pure cells by employing tissue extracts, a possibility which we will probably investigate.

The suggestions of Dr. Sabin are appreciated and will certainly be considered. We did not try to filter our supernatant fluids. We only took the tissue of the cultures, ground it with sand in a fluid medium, and centrifuged the extract at a relatively low speed, occasionally at 10,000 rpm, but most of the time at about 1,000 to 2,000 rpm. I do not know if cells were present in the supernatant fluid, but since we were using normal cells absolutely free of virus, we thought that, even if cells were inoculated, the virus would be revealed by bioassay.

Virus of Avian Myeloblastosis. XVI. Kinetics of Cell Growth and Liberation of Virus in Cultures of Myeloblasts [1, 2]

G. S. BEAUDREAU, C. BECKER, T. STIM, A. M. WALLBANK, *and* J. W. BEARD,[3] *Department of Surgery, Duke University Medical Center, Durham, North Carolina*

There have been reported from time to time (*1–6*) the results of studies of tissue cultures of myeloblasts from chickens diseased with the avian myeloblastosis virus. It has been observed that the cells possess the capacity to multiply rapidly under suitable conditions (*4–6*) and to liberate myeloblastosis virus into the culture medium. Such cells also exhibit pronounced ultrastructural characteristics (*3–6*) related to intracellular virus synthesis. For many reasons, as will be seen, these cultures of myeloblasts constitute a system of unusual value for the study of virus-host cell relationships with respect to the factors governing the synthesis and liberation of virus and those influencing induction of the state of malignancy. In consequence, much attention has been directed toward the problem of developing conditions of tissue culture suitable for supporting multiplication of the myeloblast.

Until recently, the behavior of the cultures was subject to rather wide fluctuations, with respect both to the rate of cell multiplication and to the rate of virus liberation. As the result of continued investigations, however, there has been devised a culture medium (*6*) containing growth and nutritional elements greatly effective in the stabilization of the behavior of the cultures over indefinite periods of study. In this system, the rates of cell multiplication and of virus liberation are relatively uniform and predictable within the limits of variation of the potentialities of cells from different individual chicken sources.

It is the purpose of this report to describe some of the more prominent features of cell growth and virus liberation under the best conditions thus far observed. The ultrastructural manifestations of the cells developing in the same and related cultures will be described in an accompanying publication (*7*).

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[1] Presented at a Symposium on the Phenomena of the Tumor Viruses, New York, N.Y., March 25 and 26, 1960.

[2] This work was aided by research grants to Duke University from the National Cancer Institute, National Institutes of Health, Public Health Service; from the American Cancer Society, Inc., and by the Dorothy Beard Research Fund.

[3] The authors acknowledge their indebtedness to Dr. W. M. Fairbank for aid in the development of the mathematical treatment of the data.

MATERIALS AND METHODS

The basic procedures for establishing and maintaining cultures of myeloblasts have been essentially the same since the beginning of the work with these cells (*1*). Myeloblasts were obtained from the circulating blood of chickens diseased with the myeloblastosis virus designated as the BAI strain A (*8*). In most of the experiments, the donor birds were White Leghorns of line 15 (*9*) which are highly susceptible (*10*) to this agent. These birds respond with a high incidence of disease and, in consequence, afford a relatively high percentage of potential donors for a given study. Chickens of this line, inoculated intravenously at 5 to 10 days of age with doses of about 2×10^{10} virus particles in 0.1 ml. inoculums, respond within 10 to 20 days with the appearance in the circulating blood of myeloblasts in numbers as great as 2×10^6 cells per mm.^3 Blood was drawn by heart puncture into tubes containing heparin to prevent coagulation, and the myeloblasts were recovered by centrifugation for 10 minutes at 150 × g. The yield of blood was about 5 to 15 ml., of which the volume of primitive cells (myeloblasts) was about 1 to 4 ml. Donors were selected by the results of daily examination of blood smears.

The myeloblasts were pipetted from the red cell layer and, in the most recent work, suspended in medium 199 (*11*) in a proportion of about 1 ml. cells in 10 ml. of suspension. With repeated centrifugation, the cells were washed in 3 successive changes of medium 199. On the basis of the packed-cell volume, noted in the graduated centrifuge tubes used in the washing process, the final cell suspension was parceled in lots depending on the requirements of the experiment. By repeated counts it was estimated that 1.0 ml. of cells, packed in the centrifugal field described, represented about 2.5×10^9 cells.

Cultures were established by introducing 0.05 ml. of packed cells into 5.0 ml. of culture fluid in 50 ml. Erlenmeyer flasks which were then closed with rubber stoppers. The cell concentration was thus approximately 2.5×10^7 cells per ml. of medium. The cultures were incubated at 37° C. on a rotary shaker (*1*) oscillating at 70 to 80 cycles per minute. Changes of culture fluid were made at intervals of 2 to 4 days. For this the cell suspensions were pipetted into 15 ml. graduated centrifuge tubes and spun at 150 × g for 10 minutes. The spent medium was removed for storage at −20° C., and the cells were resuspended in 5.0 ml. of fresh medium for further incubation as before.

Division of cultures was made when the number of cells was approximately 5×10^7 per ml. For this procedure, the cells of only 2.5 ml. of the culture were sedimented and then resuspended in 5.0 ml. of fresh medium. The remaining cells could be used for another culture or discarded. It was of considerable importance for continuity of the various characteristics of the cultures to maintain the concentration of cells at levels of about 2.5×10^7 to 5.0×10^7 per ml. The rate of cell proliferation was related to the conditions of culture, and the intervals between divisions varied accordingly.

Two measurements were made on the cultures at appropriate intervals: (1) enumeration of viable cells, and (2) determination of the number of myeloblastosis virus particles in the culture fluid. For the cell counts, aliquots of the well-mixed cells in culture were diluted with a preparation of trypan blue for identification of viable cells (*1*), and counts were made with a hemocytometer. Rapid enumeration of virus particles in small volumes of the culture fluids was made by a photometric procedure (*12*) for measurement of the adenosinetriphosphatase activity (*13–15*) associated with the virus particle. The standardization of the technique for accurate representation of virus particle number was controlled, as formerly (*1*, *2*), by direct particle count with the electron microscope at suitable intervals. On the basis of this comparison of particle number with activity, the enzyme unit was chosen, as a matter of convenience, to correspond, on the average, to 9.15×10^8 particles per ml. of test preparation, of which a 50 λ sample gave a reaction time of 1000 seconds.

The criteria of the processes occurring in the cultures were concerned, in this report, with the rate of cell growth and the rate of liberation of virus in terms of virus particles per cell per hour. The data describing cell growth represent the number of viable cells derived at a given time from 1.0 ml. of the initial culture. Ideally, these values would have been the sum of the respective values obtained by counts on all derivative cultures. It was not possible, however, to keep all the cells, and, in consequence, the actual data, obtained by counting the cells in a given culture, were adjusted by a calculation of the number of cells which would have been derived at the time of the count had all cultures been retained. This was accomplished by the multiplication of the number found in 1.0 ml. of the representative culture by the total number of cultures which may have existed had no cells been discarded. Virus liberation is expressed in an analogous way as the total virus derived from 1.0 ml. of the starting culture. The number of virus particles per ml. was measured in the spent culture fluids at each change of medium, and the values were accumulated from the preceding culture cycles. The resulting values were adjusted to account for the cells discarded as in the instance of the data on cell growth.

RESULTS

The experiments presented here have been selected for exemplification of the major principles of cell growth and virus liberation observed in the study of myeloblasts during the past 3 years. Whereas wide variations encountered in the earlier experiments interfered with interpretation of the results, it is now possible by virtue of the findings to be described to perceive some elements of the trend of potentialities of the cells both for growth and for the synthesis and liberation of virus. The data are such that they are susceptible to mathematical interpretation, and analysis has been made of the data observed with the principal types of experiments.

Cell Growth and Virus Liberation in Cultures of Myeloblasts Fresh From the Chicken and Established in 50 Percent Chicken Serum in Medium 199 Supplemented With Added B Vitamins

The development of the culture medium currently in use has been guided by the results of numerous studies for various periods after preparation of cultures *chiefly with cells fresh from the circulating blood.* On the basis of the findings in work to be described in a later report, it was found that a critical requirement for the stabilization of cell growth was the presence in the medium of relatively high concentrations of *folic acid.*

The medium that has thus far yielded the best results consists of a mixture of equal parts of normal chicken serum and a modified form of medium 199. The modifications consisted in the increase in the concentration of (1) glucose from 1.0 to 5.0 gm. per liter; (2) the B vitamins (biotin, pantothenic acid, pyridoxal, thiamine, nicotinamide, and riboflavine) to the concentrations found in Eagle's (*16*) supplement; and (3) folic acid to 0.8 mg. per liter. Antibiotics were added to the whole medium in all experiments in concentrations of 50 units of penicillin and 50 μg. of streptomycin per ml. The serum was always heated for 30 minutes at 56° C. before preparation of the mixture.

The results of a typical experiment made with this medium are shown in text-figure 1. It is evident from inspection that cell proliferation conforms closely to a typical exponential growth rate function. This rate of growth was not attained immediately, but after a delay of approximately 4 days, after which the rate expression was linear throughout the remainder of the 30-day study.

Virus was first measured in the initial cultures after 3 days, and thereafter the agent was liberated at a rate apparently related to the number of cells present. Analysis of the data indicates that the rate of virus liberation, that is, the number of virus particles per cell in unit time, was constant in this experiment. This can be expressed by equation (1),

$$\frac{dV}{dt}=k_vN \qquad (1),$$

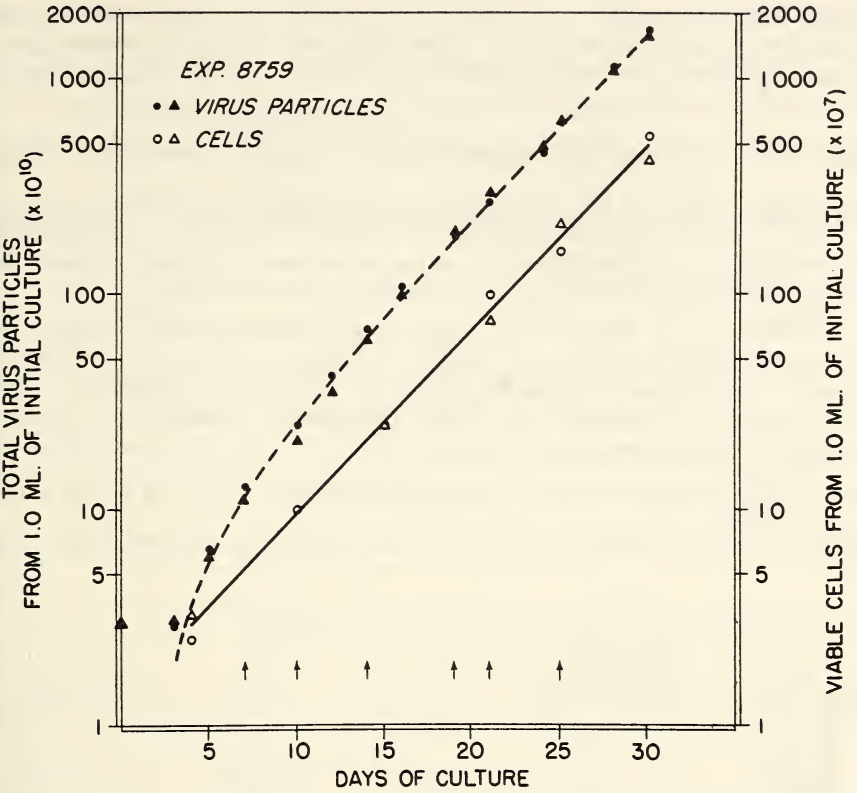
in which V = the total number of virus particles at time, t, and N = the number of viable cells at time, t. The virus liberation constant (virus particles/cell/hour) is represented by k_v.

The equation describing the exponential rate of cell growth is

$$N=N_oe^{\lambda t} \qquad (2),$$

where N_o = number of viable cells at the beginning of measurement; λ is the cell growth constant; and N = the number of cells at time, t. Substitution of N of equation (2) for N of equation (1), yields the relationship

$$\frac{dV}{dt}=k_vN_oe^{\lambda t} \qquad (3).$$



TEXT-FIGURE 1.—Cell growth and virus liberation in a culture of myeloblasts freshly obtained from the blood of a chicken with myeloblastosis. The culture fluid was 50 percent chicken serum in medium 199, with added glucose, B vitamins, and a concentration of 0.4 μg. of folic acid per ml. of whole medium. The *continuous* line was drawn by the method of least squares through the observed points representing cell growth. The *dash* line represents the expected total virus calculated by use of equation 4. Division of the cultures was made at the points indicated by the *arrows*.

On integration of equation (3), there is obtained the expression

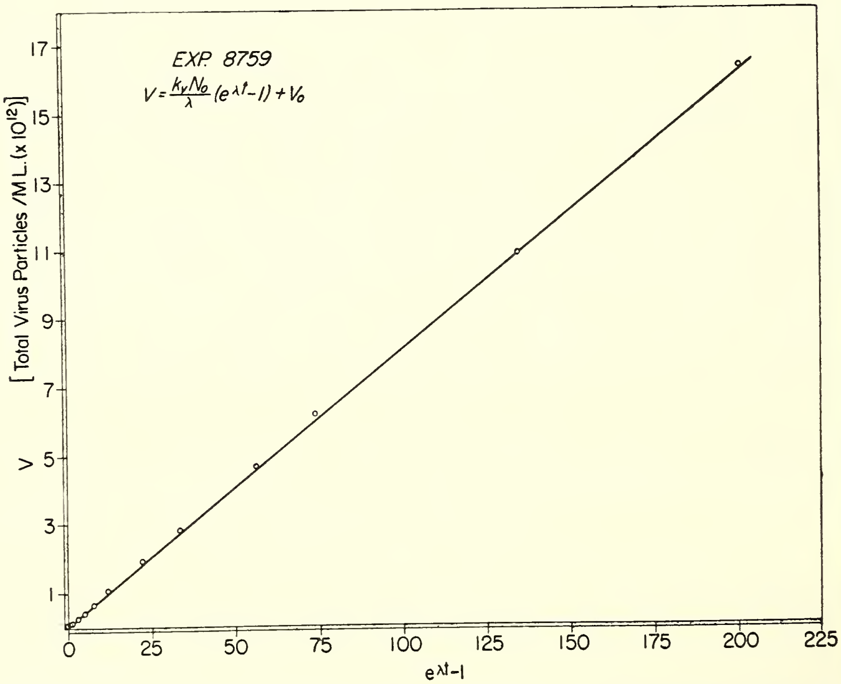
$$V = \frac{k_v N_o}{\lambda}(e^{\lambda t} - 1) + V_o \qquad (4),$$

in which V_o represents the number of virus particles present when $t = 0$.

When no virus is present at the beginning of the period of measurement, that is, when the cells have been washed free of the agent, $V_o = 0$, the expression applicable is $V = \frac{k_v N_o}{\lambda}(e^{\lambda t} - 1)$ (5).

The applicability of these equations for interpretation of the results of the experiments with the myeloblasts can be tested by the fit of the observed data of experiment 8759 to the theoretical relationship. In text-figure 1 the continuous straight line was drawn by the method of least squares through the points representing the experimental data obtained by periodic counts of viable cells made during the period of exponential growth extending from 4 to 30 days. From the equation of this line the slope of the regression b_c, $\left(b_c = \frac{\log \frac{N}{N_o}}{\text{days}}\right)$ was 0.0853. From this value, $\lambda \left(\lambda = \frac{b_c}{\log e \cdot 24}\right)$ was found to be 0.00819 hours^{-1}. The number of cells, N_o, at the beginning of exponential growth obtained from the intercept at 3 days, was 2.41 × 10^7 cells per ml. of culture.

Equation 4 is such that a plot of the experimentally determined number of virus particles, V, against the function $(e^{\lambda t} - 1)$ should be linear. The results of this treatment of the data of experiment 8759 are shown in text-figure 2, in which the points represent the observed number of virus particles at various values of t. The line was drawn by the method of least squares. The slope of this line, $\frac{k_v N_o}{\lambda}$, was 81.06 × 10^9, and k_v was thus found to be 27.6 virus particles per cell per hour. V_o,



TEXT-FIGURE 2.—Total virus particles derived from 1 ml. of initial culture plotted according to equation 4. The *open* circles represent experimental values. The *continuous* line was drawn by the method of least squares, and the slope, $\frac{k_v N_o}{\lambda}$, is 81.06 × 10^9. From this, k_v = 27.6 particles per cell per hour. The intercept, V_o, of the line is at 19.3 × 10^9 particles.

the intercept of the line, was 1.93×10^{10}. The various values are cited in table 1.

The constants, λ and k_v, determined in this way were used in equation 4 for calculations of the expected number of virus particles at different times, based on the original assumption that $\frac{dV}{dt} = k_vN$, to yield the curve indicated by the dash line in text-figure 1. It is evident that the calculated curve is a very close fit to the experimental data. Thus, it may be concluded that the rate of virus liberation was constant and was 27.6 virus particles per cell per hour. The generation time of the cells was 3.5 days (table 1).

TABLE 1.—Cell growth and virus liberation constants of cultures of myeloblasts and a comparison with analogous data derived from results of studies on the Rous sarcoma by Prince (*17*)

Experiment	b_c $\left(\frac{\log \frac{N}{N_o}}{\text{days}}\right)$ Days^{-1}	λ $\left(\frac{b_c}{\log e \cdot 24}\right)$ Hours^{-1}	k_v $\left(\frac{\lambda V}{N_o(e^{\lambda t}-1)}\right)$ Particles · cells^{-1} hours^{-1}	Cell generation time $(\log 2 \cdot b_c^{-1})$ Days
8759	0.0853	0.00819	27.6	3.5
11157				
Flask 6Z	0.0385	0.00370	25.0	7.8
" 6AM	0.0438	0.00420	26.0	6.8
" 6AN	0.0380	0.00365	20.4	7.9
" 6AO	0.0430	0.00412	21.5	7.0
Mean	0.0408	0.00392	23.2	7.4
10258	0.0831	0.00797	19.5	3.6
RSV*	0.280	0.02692	3778 †	1.1

*RSV = calculations from the data of Prince given in figure 5 of (*17*).

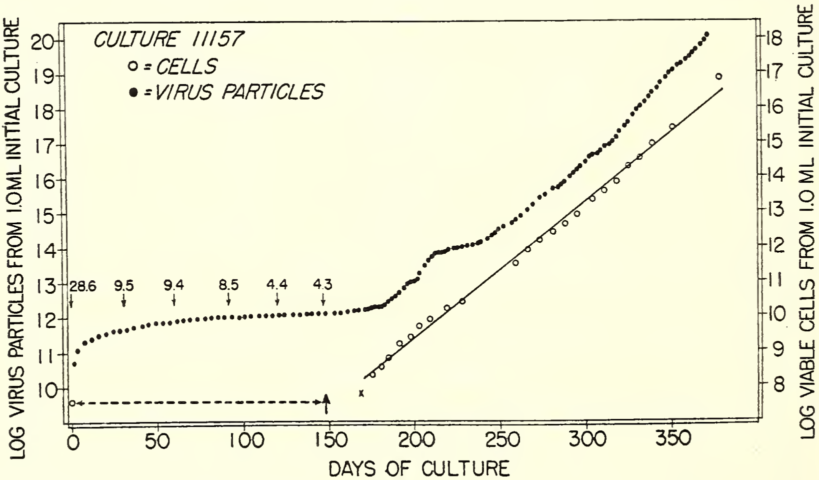
†Pock-forming units per mg. dry weight per hour.

Variation in Behavior of Cells on Protracted Culture in Different Media

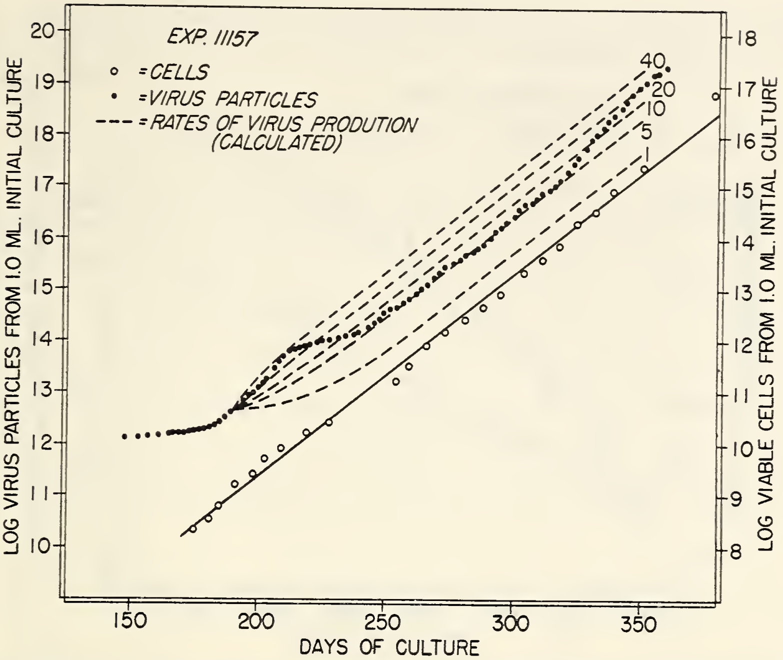
In the earliest experiments (*1*) the culture medium was 20 percent chicken serum and Gey's salt solution. The cells could be maintained for long periods in a state in which the rate of cell proliferation balanced approximately the rate of cell death. In this medium, virus was liberated first at a high rate followed by a gradual decline in the rate. In the first part of the curve of text-figure 3, it is seen that the rate of virus formation decreased from 28.6 to a level of 4.3 particles per cell per hour during the course of the study period of 148 days, while the cell population remained essentially constant. On the 148th day the cells were put into a mixture of equal parts of chicken serum and medium 199 *without B vitamin supplement.* Within a few days, an increase in cell population was noted, and on the 169th day the culture was divided. The course of cell proliferation thereafter is indicated by the increase in the number of viable cells. The fit of the experimental points to the straight line drawn through the observed points by the method of least squares showed that the rate of cell growth in the study period from 175 to 379 days was essentially exponential. This line represents a generation time of 7.7 days.

In contrast, the values representing virus liberation exhibited large and erratic variations. The nature and degree of these variations between 190 and 365 days can be seen from the relations derived by analyses similar to those of the preceding section and illustrated also in text-figure 4. The dash lines ($t = 0$ on day 190) represent the expected relations on the assumption of constant rates of 40, 20, 10, 5, and 1 virus particles liberated per cell per hour.

The results of text-figure 4 show that, whereas the medium supported an exponential rate of growth of the cells, the rate of virus synthesis and liberation, nevertheless, varied greatly. Although the exact basis for this variation was not discernible, it was realized that some or much of it was related to the use of different batches of serum during the study period (*1*). In such a system, however, it could not be predicted in advance that the variation might not be due to actual changes in the properties of the cells due to prolonged maintenance in culture. That this was probably not the case was shown by the results of the following study.



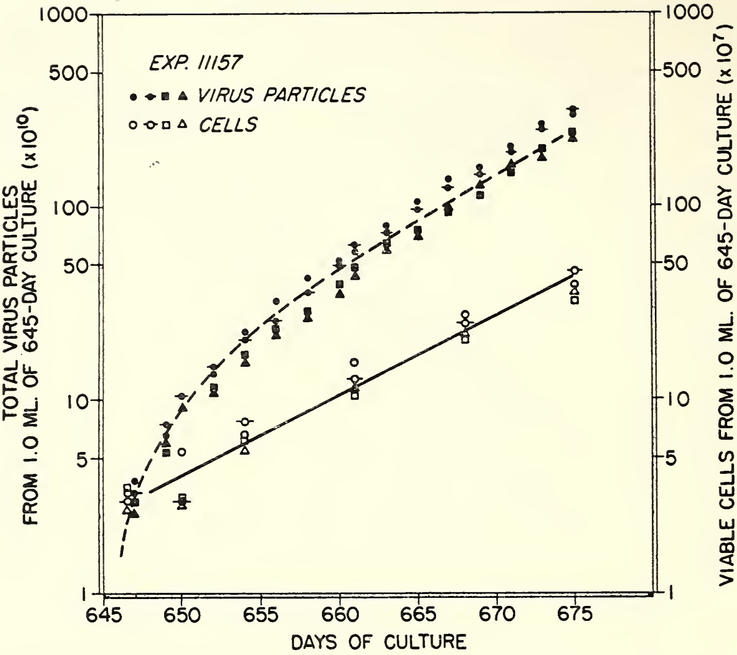
TEXT-FIGURE 3.—Behavior of a long-term culture of myeloblasts, first (*1*), in a medium of 20 percent chicken serum in Gey's salt solution for 148 days, and then, in a mixture of 50 percent chicken serum in medium 199 with added glucose but *without added B vitamins.* The *dash* line signifies lack of increase in cell population in the initial period. At 169 days (indicated by the *cross*) cell growth was sufficient to permit division of the culture, and the culture was divided 33 times thereafter. The *open* symbols represent the results derived from cell counts, and the *continuous* line was drawn by the method of least squares. The numbers above the virus data (*closed* circles) indicate rate of virus liberation per cell per hour at the intervals shown by the corresponding *arrows* from 0 to 148 days.



Text-figure 4.—Analysis by application of equation 4 of the data of a portion of the experiment of text-figure 3. The *dash* lines were drawn from day 190 to represent the relations expected on the assumption of constant rates of virus liberation of 40, 20, 10, 5, and 1 particles per cell per hour.

Stabilization of Cell Proliferation and Virus Liberation of Long-Term Cultures of Myeloblasts Introduced Into Vitamin B-Supplemented Medium

The cells used in this experiment were those derived from the same cultures described in the preceding section. On the 577th day of culture, the medium was changed to the mixture of equal parts of chicken serum and medium 199 *supplemented with additional amounts of B vitamins, including the high concentration of folic acid.* The results observed during the study period between 645 and 675 days are shown in text-figure 5. Here it is seen that the pattern of cell proliferation and virus liberation observed with these cells derived from long-term culture under highly varied conditions is identical with that of cells freshly obtained from the bird. The rate of cell growth was exponential with a generation time of 7.4 days. With the use of the relations of equation 5 in the manner described for experiment 8759 and the mean constants given in table 1, the calculated dash line was derived as shown. As can be seen, the agreement between the calculated line and the experimental points is excellent. From this it can be concluded that the variations occurring in the liberation of virus by these cells under the different conditions prevailing before must have been related to deficiencies in the medium.



Text-figure 5.—Cell growth and virus liberation in a later period, 645 to 675 days, of the same long-term culture established as shown in text-figure 3. Beginning on the 577th day, the culture medium was 50 percent chicken serum in medium 199 with added glucose and B vitamins and a concentration of folic acid of 0.4 μg. per ml. of whole medium. The *continuous* line was drawn through the cell data by the method of least squares, and the *dash* line was constructed by application of equation 5.

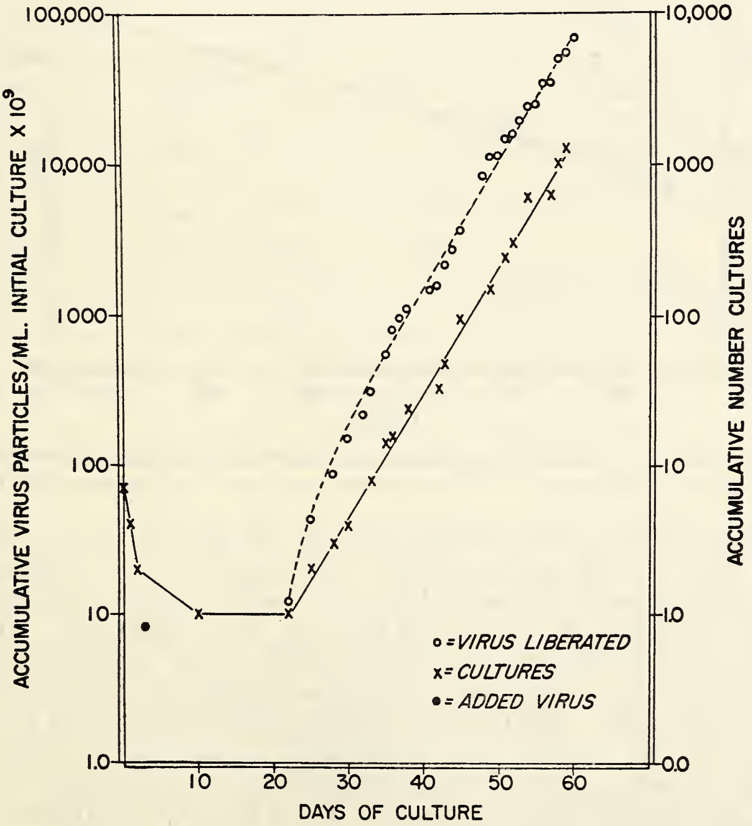
Myeloblasts Derived by Treatment of Normal Bone Marrow Cultures With Myeloblastosis Virus

In an earlier report (*5*) there was described the proliferation of myeloblasts in cultures of normal bone marrow exposed to the myeloblastosis virus. The medium used in these studies was 50 percent chicken serum in medium 199 *without B vitamin supplement.* Analysis has been made here of the results of one of the experiments of the series, text-figure 1 of (*5*). Measurement of cell growth in that study was made not by cell count but on the basis of the number of cultures accumulating during the study period. The constants derived for cell growth and virus liberation are given in table 1. The data of the experiment are charted in text-figure 6.

Here it is evident that, from the time of the beginning of cell growth at 22 days, the rate of proliferation was constant with a generation time of 3.6 days. Again, as in the other types of experiments, the observed data on virus liberation conformed closely to the theoretical, represented by the dash line derived by use of equation 4. The rate of virus liberation was 19.5 particles per cell per hour. In principle the behavior of the myeloblasts emanating from normal bone marrow exposed to virus *in vitro* was identical with that of myeloblasts from diseased birds established in culture.

Analyses of Rous Sarcoma Data From Studies by Prince (*17*)

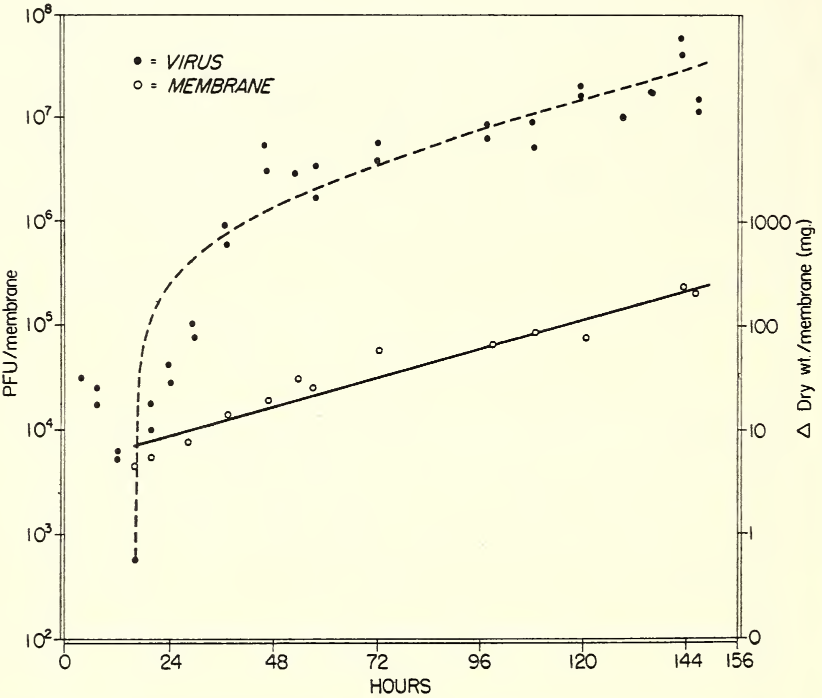
It would seem of interest to compare the results observed with the myeloblast system with those of analogous studies by Prince (*17*) on cell growth and virus formation in the chicken chorio-allantoic membrane inoculated with the Rous sarcoma virus. Although the conditions of the respective studies were entirely different, the analyses have revealed some points of similarity in the behavior of the two systems. The data were obtained from figure 5 of Prince's report. In his studies, virus increase was recorded in terms of pock-forming units (infectious units of virus), and the rate of cell growth was determined as increase in dry weight of the virus-inoculated membrane. The line through the growth points in text-figure 7, in our report, was drawn by the method of least squares,



TEXT-FIGURE 6.—Growth and liberation of virus by myeloblasts derived from normal bone marrow exposed to myeloblastosis virus in culture (expt. 10258). The analyses were made by use of equation 4 as in the experiment of text-figure 1 (*see* text).

and, with the constants cited in table 1, calculation was made of the theoretical relationship concerned with virus formation as indicated by the dash line.

In text-figure 7 it is seen that the experimental data, with respect to virus extractable from the growths in the membranes, correspond closely to the theoretical values in the period from 36 to 144 hours of study. It may be concluded, then, that in this period of activity, the rate of Rous sarcoma virus formation per increase in membrane mass per hour was constant.



TEXT-FIGURE 7.—Analyses of the data on Rous sarcoma virus obtained by Prince (*17*) by application of equation 4. The constants used were those given in table 1.

Decrease in extractable virus was noted in the initial stage of 16 hours, after which a rapid increase ensued. The observed rate of increase of extractable virus in the period between 16 and 36 hours, in relation to increase in membrane weight, was less than the expected rate. This is contrary to the interpretation of Prince, who considered the rate of increase in this interval as greater than the increase occurring in the later periods of the experiment. Whether the deviation from the theoretical was real because of the complexities of participation of two types of tissues in tumor formation, or due to technical difficulties of measurement in the early stages of growth is not apparent. It can be seen, nevertheless, that, as observed with the myeloblast culture system, the rate of virus formation in relation to increase in the mass of tumor was constant through a large part of the study.

DISCUSSION

The findings described here serve to demonstrate two outstanding biologic characteristics of the primitive cells of malignant attributes occurring in virus-induced myeloblastosis: the potentiality (1) to proliferate at exponential growth rates *in vitro* and (2) to liberate the myeloblastosis virus at a constant rate in a given culture series. Attainment of this status of the myeloblast culture system has been dependent on the increasing recognition of the requirements of the cells with respect both to their own growth processes and, as well, to the activities concerned with the synthesis of virus.

With respect to cell growth, the myeloblasts possess fully those potentials for proliferation which enable them to multiply at high constant rates *in vitro*. The results were the same with myeloblasts freshly obtained from the blood of diseased chickens; with those derived by treatment of normal bone marrow with virus *in vitro*; or those held for long periods in culture under highly varied conditions. This property of the cell could not be predicted in advance and has been appreciated, finally, only by the evolution of a medium capable of supplying consistently the nutrient and growth factors required for the stabilization of the growth processes involved. In its capacity to proliferate in culture, the myeloblast associated with the avian myeloblastosis virus differs profoundly from the myeloblast of the normal bone marrow, which does not survive in culture under the conditions thus far investigated (*5*). Thus, the proliferative capacity of the virus-associated cell in culture constitutes an outstanding biologic criterion of the influence of the agent.

Myeloblastosis in the intact bird is a highly malignant disease, in which the only manifestation of neoplasia is the tremendous overgrowth of myeloblasts. Other elements, such as reticular cells of the bone marrow and spleen, macrophages, and endothelial cells, are obviously susceptible to infection, but only the myeloblasts display neoplastic traits. These characteristics are not lost in culture, and, furthermore, they are evident in cells emanating from normal bone marrow exposed in culture to the myeloblastosis virus. Studies have not yet been made to determine whether every virus-exposed cell is malignant or is actually infected with the virus. It is not too remote to assume, however, that for practical purposes all the myeloblasts in the blood of the diseased bird and those in culture are virus-infected. This is based on the previously mentioned limited capacity of the normal myeloblast to proliferate, either in the bird or in culture. Thus, cells not under the influence of the virus would be expected to mature to granulocytes in the bird or to succumb in culture. Furthermore, there can be seen in thin sections, under proper conditions (*3*, *5*), evidence of virus in essentially every cell.

From these considerations it may be concluded that the myeloblasts *in vitro* constitute essentially pure cultures of virus-induced malignant cells, which can be maintained indefinitely in a state of exponential growth. The cells occur as individuals in suspension in the blood of the host from which they can be procured as wished and quantitated by the simple process of enumeration in a hemocytometer. In addition, the cells grow best in suspension in shaking cultures which provide the same advantages for quantitation. With these assets, the cultures comprise a practically ideal material for the study of the factors governing the growth of a virus-induced tumor cell.

The factors influencing growth of the myeloblasts have not yet been fully defined. The usefulness of the culture system for extensive and controlled studies has depended on the stabilization of growth behavior. Aside from the supplementary nutritive and growth factors supplied by medium 199, the essential constituents consist of chicken serum in 50 percent concentration and the very high concentration of folic acid. As already seen, exponential cell growth and constant rates of virus liberation have been observed (*4*) in a medium of whole serum and, in other studies (*5*), in a medium of 50 percent chicken serum in medium 199 without additional folic acid supplement. This was a rare experience, however, because of great variation in the efficacy of different batches of serum (*1*). With folic acid, the behavior of the cultures has remained uniform. Some information has been gained of the amino acid requirements, and studies have been made of the influence of B vitamins other than folic acid. The basis of the need for high concentrations of serum is not known, but it is clear that no substitute for it has been found. Fortunately, serum collected easily and economically in large volumes at a chicken abattoir yields entirely satisfactory results with the addition of the high concentrations of folic acid. The results of studies on the evolution of the present medium will be discussed in detail in a later report.

The phenomena of synthesis and liberation of the myeloblastosis virus by myeloblasts in culture have been the primary stimulus to the continued efforts to grow the cells under controllable conditions. Some of the most important fundamental questions, concerned with the virus tumor problem in current work, are those pertaining to the factors governing the onset and maintenance of cell infection. For the study of such virus-host cell relationships the myeloblast culture system seems thus far without equal. The characteristics of cell growth have already been described. The agent can be titrated rapidly and accurately by virtue of the enzyme activity to dephosphorylate adenosine triphosphate. Furthermore, this assay measures, not infectious units, but absolute number of virus particles. In this respect, it is a fortunate circumstance that the enzyme activity is independent of the infectious moiety of the virus (*18*), and is highly stable under the conditions of tissue culture. The results of the assays are interpretable with complete assurance as representative of the output of virus in terms of actual particles, as can be fully corroborated by direct counts with the electron microscope. Of equal significance is the occurrence, under proper conditions, of ultrastructures specific to the virus-infected cell. These, obviously associated with virus synthesis and liberation, are of a size and other characteristics suitable not only for study by electron microscopy, but by the methods of cytochemistry and other analyses with the phase and light microscope (*19*).

The principal biologic criterion of the virus-cell relationship thus far employed has been that of the rate of virus liberation. Although there is little evidence yet of the precise nature of the factors which govern the rate of liberation, the accumulated data provide the basis for limited conclusions. In the present work, it was seen that, under the prescribed conditions, the rate of virus liberation per cell per hour was constant in a given experiment and was thus precisely in parallel with those cellular processes resulting in exponential growth of the myeloblasts. This occurred in cells freshly drawn from the host and those recovered from a culture series maintained for almost 2 years *in vitro*. It is evident, table 1, that the rates of cell growth (generation time) were not the same with cells from different sources. Like variations occurred in the rates of virus liberation, but the respective rates of cell growth and virus liberation did not vary in parallel. This principle is well illustrated by the results of experiment 11157 (text-fig. 3) in which the cells were in a state of exponential growth for 204 days. During this period, however, the rate of virus liberation fluctuated through an approximately eightfold excursion from 40 to 5 and back to 30 particles per cell per hour. The events in the earlier stages of the experiment in which the cells were in a mixture of 20 percent chicken serum in Gey's salt solution can only be surmised, since cell counts were not made. To judge from the cell volume observed with each medium change, the population of cells during this period was essentially constant. Calculations made on this assumption showed, as was seen in numerous other experiments (*1*), that the cells fresh from the bird liberated virus at a high rate in the early period of culture and that the rate declined soon to a level of less than 5 particles per cell per hour. Despite the low level of proliferative activity of the cells under these conditions, virus was still liberated at a rate comparable with that observed with derived cells in a phase of rapid multiplication.

From what has been seen thus far, it may be judged that the processes concerned with virus synthesis are independent of those involved in cell multiplication, at least in the sense that they can proceed at different rates and are by no means wholly regulated by the same metabolic activities. This is but a particularly nice and quantitive corroboration of relations which have been suspected or indicated for other virus tumors in the host. It is well known, for example, that the virus yields from rapidly growing cottontail rabbit warts vary greatly (*20*), and the same is seen with the Rous sarcoma, in which quantitative studies (*21*) of thin sections have revealed a relationship between intracellular and extractable virus but none between tumor virus content and rate of tumor growth.

The absolute status of the myeloblast culture system with respect to the ultimate capacity of the cells to multiply and to synthesize virus cannot be fairly assessed at the present. The highest rates of cell proliferation have been a doubling time of approximately 3.5 days. The highest rates of virus liberation have been about 40 particles per cell per hour in cultures in which cell growth was exponential. Under the best conditions the rate of virus synthesis has been constant in the presence of exponential growth of the cells. It is already known that the rate of virus release is subject to control by the environment of the cell. It remains to be seen whether the processes of virus synthesis can be terminated in the intact cell.

The expressions employed for analysis of the myeloblastosis data have been applied to results obtained by Prince (*17*) relative to virus growth in the chick chorio-allantoic membrane inoculated with Rous sarcoma virus. The results observed in the latter stages of short-term studies with this system were analogous in principle to those with the myeloblast system with respect to a constant rate of formation of Rous sarcoma virus in relation to increase in the mass of the virus-inoculated membranes.

To summarize: Earlier studies have shown that, under some conditions, the myeloblasts of avian myeloblastosis may proliferate at exponential rates in tissue culture, in one instance approximately 500 days, and consistently liberate myeloblastosis virus into the culture medium. In the present work, analyses have been made to clarify some aspects of the kinetics of virus liberation and thus, also, of virus synthesis in association with the cell. The analytical treatment has shown that in some cultures proliferating exponentially for long periods, the myeloblastosis virus was liberated at constant rates in terms of absolute number of virus particles per cell per hour. This activity of the cell can be described by a simple mathematical expression which yields constants applicable to the interpretation and comparison of data from experiments of various types. Application of these analyses has revealed the identity of the principles of behavior of myeloblast cultures of various derivation, such as cells freshly obtained from the circulating blood of birds with myeloblastosis, myeloblasts carried for periods of nearly 2 years under various conditions of culture, and myeloblasts derived by treatment of normal chicken bone marrow with the virus *in vitro*. In the studies under consideration, the generation times varied from 3.5 to 7.7 days, and the rates of virus liberation were within the range from about 5 to about 40 virus particles per cell per hour. The variations in the rates of virus liberation did not parallel those of the growth rates of the cells in different phases of study. From these findings the interpretation may be made that the respective metabolic processes of virus synthesis and cell growth are not inflexibly coupled and thus may vary independently. The results have served to emphasize the stabilizing influence of balanced culture media consisting of 50 percent chicken serum in medium 199 supplemented by B vitamins and folic acid in high concentration.

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Discussion

Dr. Rubin (University of California): Experiments have been published on the growth rate of Rous sarcoma virus (RSV) in cells removed from chicken sarcomas and maintained in tissue culture. (Rubin, Virology 1: 445, 1955; Rubin, Ann. New York Acad. Sc. 68: 459, 1957). It was reported that RSV is produced at a constant rate which is proportional to the number of cells and the cell mass. Equations were developed to describe the quantitative relationship between virus and cell under conditions of a static cell population and exponential cell growth. The latter equations are similar to those introduced by Beaudreau *et al.*, but they take into account thermal inactivation of RSV since the assay relies on infectivity. In the present case when the assay is based on particle counts this would appear to be unnecessary. However, if we make the likely assumption that the particles can readsorb to the cells, then we must compensate with a term similar to that for heat inactivation. This is particularly true in view of the high cell concentration used in Beaudreau's experiments, which would almost assure rapid loss of particles through readsorption.

These equations are not applicable to experiments which are concerned with the time course of infection after exposure of normal cells to RSV (Prince, Virology, 5: 435, 1958; Temin and Rubin, Virology, 8: 209, 1959). In the latter instance, the initial virus growth is exponential, as it is in any other virus infection. It only levels off to a constant rate after 3 or 4 days. In this regard RSV infection is like influenza virus infection. In fact, the growth curves of the tumor viruses which have been precisely studied are qualitatively indistinguishable from the growth curves for the myxoviruses. They differ only quantitatively, particularly in having a longer latent period and in producing infectious virus at a slower rate.

The comparison of intracellular and extracellular virus in both the established tumor (Rubin, Ann. New York Acad. Sc., 1957) and the freshly infected cells (Temin and Rubin, Virology, 1959) established that RSV is continuously released from cells as soon as the newly synthesized particle gains the property of infectivity, which suggested that, like influenza virus, RSV is completed at the cell membrane.

We have recently been able to show that the initial exponential shape of the RSV growth curve is due, at least in part, to an asynchrony in the time at which individual infected cells start to release virus. The morphologic alteration of chicken fibroblasts into Rous sarcoma cells is associated, at the population level, with the onset of virus production, and may be a direct consequence of virus production. Of particular interest is the change in the cell surface which must accompany the multiplication of any virus that is completed at the cell surface. Cell surface changes are likely to play a critical role in releasing the cell from contact inhibition (Abercrombie *et al.*, Exper. Cell Res. 13: 276, 1957) and thus making the cell a cancer cell.

The evidence in Dr. Beaudreau's paper for exponential cell growth should be supported by experiments in which the increase of cells in any *individual* passage is measured over at least a tenfold range, rather than the twofold range employed. One cannot establish exponentiality by transferring the contents of 1 dish to 2 dishes to 4 dishes, etc., because one is then only measuring the exponentiality of the manual operation of transfer. From general experience with other cells, it would be surprising to encounter exponential cell growth at cell concentrations from 2.5 to 5×10^7 per ml. It is quite rare, even with cell lines well adapted to grow in tissue culture, to get exponential cell growth beyond a concentration of 10^6 cells per ml.

The use of particle counting to determine virus concentration has disadvantages as well as the advantages indicated in Dr. Beaudreau's paper. The chief disadvantage is most strikingly brought out by our recent finding (Rubin, unpublished) that some normal chick embryos are heavily infected with a virus which, in its biologic behavior *in vitro*, resembles lymphomatosis virus. We detect this virus because it induces resistance in cells to infection by RSV, if it has a head start—hence, its designation RIF for resistance-inducing factor. This agent is physically and immunologically indistinguishable from RSV, but, of course, does not produce the morphologic alteration which characterizes RSV infection. It appears to be ubiquitous in distribution—it is even present in our stocks of RSV. Any investigation of chicken tumor viruses which relies on particle counting will have to take this agent into account. Unless a precise relationship is established between particle count and biologic effect, and this relationship is demonstrated to remain constant throughout the course of an experiment, the particles being counted may be something quite distinct from the biologic problem under investigation.

Dr. Beard: In one principal aspect, the avian myeloblast culture system differs greatly from that of the Rous sarcoma discussed by Dr. Rubin. The myeloblasts established in culture were obtained from chickens with virus-induced myeloblastosis, and, as will be indicated in Dr. Bonar's paper later, it is probable that everyone of these cells carries the virus.

What Dr. Beaudreau has shown is that a system has been devised in which a virus-induced malignant cell will grow at an exponential rate over indefinite periods. Furthermore, the myeloblast is a natural host cell of this virus, both in the chicken and under artificial conditions *in vitro*. In this respect the myeloblast system is similar to that of the mouse mammary carcinoma studied by Dr. Lasfargues.

As a second principal feature of the myeloblast system, it was shown that the cells liberate the specific virus at a constant rate under the prescribed conditions throughout the period of study.

Dr. Beaudreau's paper has thus showed the recognition of a system of unique value for the study of the relationships between virus and cell in the induction of malignancy and maintenance of malignancy.

Dr. Sanford (National Institutes of Health): In the system described by Dr. Beaudreau, it is important to know the rates of cell necrosis which may distort the data completely. Were these rates measured?

Dr. Alice Moore (Sloan-Kettering Institute): I should like to ask if the infection of normal bone marrow in culture resulted in a change in the morphology of the myeloblasts?

Dr. Beaudreau: In answer to Dr. Rubin's discussion it needs to be said only that the application of the equation described here was simply an attempt to evaluate the rate of product formation (virus formation in this case) with respect to the worker (the cells) in a situation in which the working population was expanding at a rate definable by an exponential relationship. This general type of equation could be used for many purposes, for example, to describe the release of radioactive material from isotope-labeled cells or, for that matter, of automobiles produced by factory workers, as long as the rate of expansion in the number of the workers can be defined and the product counted. The application of this expression to the data of Prince on Rous virus formation was made only for the purpose of pointing out the necessity for consideration of both the virus and the cell together in a discussion of *rates* of virus formation. From text-figure 7 of the paper, it can be seen that the experimental points during the early part of the infection in Prince's experiments fall below the calculated values which assumed a constant rate of virus formation. This observation may be attributed to either an increasing capacity of the cells to produce virus or, as Dr. Rubin and others have suggested, an increased number of active infected centers. It is of interest that myeloblastosis virus formation shows a reversible effect similar to this under certain nutritionally deficient conditions.

The comparison of tumor virus formation with virus agents that do not induce tumors deserves some comment. Although there may be analogous processes involved in both, the inescapable difference is that the invasion of the cell by tumor virus causes a change in the host cell which makes it durable with respect to the host animal. The nature of this durability is the center of the tumor problem.

With respect to Dr. Sanford's comment, it is evident that more information should be obtained relative to the death rate of the cells and to the cell turnover in the population. At the present time, there is not sufficient information to give an accurate answer to this question.

However, the equations proposed for this system and the data collected to resolve the equations were based on the number of viable cells in the population.

With respect to the infection of normal bone marrow cells, it must be recognized that the cell elements in this tissue are of many kinds, and it is difficult to evaluate the changes within the first 20 days. At the present time, however, it is evident that the demonstrable response of the bone marrow cultures to the virus is the growth of myeloblasts which do not differ in morphology from the blood myeloblasts, established in culture, of the diseased chicken. The myeloblasts of bone marrow infected in culture differ from normal myeloblasts in the presence of virus-specific structures. Later Dr. Bonar will discuss the ultrastructure of the myeloblast and will describe the present knowledge of the characteristics of the virus-infected myeloblast. In our experience these ultrastructures have been evident fairly constantly during the culture periods and the release of the virus. Studies are now being made during the period prior to the 20 days after which infection of the myeloblast is obvious. Electron micrographic studies show that infection can be recognized about 12 days after exposure of the bone marrow to virus.

Dr. Eagle (National Institutes of Health): From the point of view of cellular physiology and without reference to the question of virus yield per cell, I am puzzled by the concentration of cells at which Dr. Beaudreau obtains logarithmic growth.

In all systems of human and animal cells which we have studied, logarithmic growth was observed at a range of cell concentration almost 1 percent of what you have used. That would be something like 2 to 5 times 10^5 rather than 2 to 5 times 10^7 cells per ml. This is a volume-to-volume ratio of approximately 0.1 percent. At this level, the cells constitute approximately 1 part in 1000 of the fluid volume.

You have said that the myeloblast is a small cell, which could account for part of the discrepancy, but I wonder whether it accounts for all of it. Do you know the number of cells per ml. of packed cells, which, in other animal cells, is about 2.5×10^8 per ml.?

Dr. Beaudreau: Yes, 1.0 ml. of packed myeloblasts contains about 2×10^9 cells.

Dr. Eagle: From these values, there is a tenfold ratio between the myeloblasts and mammalian cells. You are therefore getting growth at a cell population density which, in terms of cell mass, is 10 times greater than that yielding optimal growth for the mammalian cell.

For this reason Dr. Sanford's question is quite relevant. Is the growth really logarithmic? If not, and only a small fraction of the cells are growing, and if there is an important degree of cell death, your values relative to virus yield per viable cell may be much smaller than they should be.

What was the criterion of the viable cell in these very concentrated suspensions?

Dr. Beard: The best estimate of the viable cell population is that at any one time the number of dead cells in these preparations hardly exceeded 5 percent.

The counts of viable cells were made with trypan blue. It is known that this does not give accurate results, but no better way is thus far available. Thus, within the limits of the methods employed, the values given are related to viable and not dead cells.

With respect to the biologic characteristics of these cells, it has not been possible to distinguish between the myeloblasts in the bone marrow and the myeloblasts in tissue cultures, except that the myeloblasts in culture all contain virus or viroplasts.

Dr. Beaudreau: In answer to Dr. Eagle's comments, one characteristic of the myeloblast, discussed in a previous publication with Dr. Becker, is the indication that the carbohydrate utilization by this cell is very efficient. No lactate accumulates in the medium during culture, since all is actually utilized by the cell. This would give a 10- to 15-fold advantage with respect to carbohydrate utilization and energy supply for cell synthesis. This advantage, together with the cell mass and the fact that the lactate concentration does not increase, may possibly account for the difference in growth levels between the myeloblasts and mammalian cells.

Growth Curves of Polyoma Virus in Mice and Hamsters[1]

WALLACE P. ROWE, JANET W. HARTLEY, JOHN D. ESTES, *and* **ROBERT J. HUEBNER,** *Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases,*[2] *Bethesda, Maryland*

KNOWLEDGE of the pattern of growth of an infectious agent is important to an understanding of pathogenesis and natural history of the infection. This report presents data on the general patterns of growth, organ distribution, and persistence of polyoma virus when inoculated parenterally into newborn mice, weanling mice, and suckling hamsters.

MATERIALS AND METHODS

Virus.—Inoculations for all experiments were infected mouse embryo tissue-culture fluids; procedures for preparation of virus stocks have been described elsewhere (*1*). Unless otherwise stated, virus used was the LID–1 strain of polyoma virus (*1*).

Animals.—Mice were of the general purpose randombred Swiss albino strain, obtained from the Animal Production Section of the National Institutes of Health; the mice in this colony have been shown to be free of polyoma virus infection as measured by absence of antibody in more than 1,700 adult mice of various strains tested (*2, 3*).

Pregnant Syrian hamsters were received from the Animal Production Section of the National Institutes of Health and from several commercial hamsteries. Serums from representative animals from each colony were tested for hemagglutination-inhibiting (HI) antibody to polyoma virus, and none were positive.

Preparation of materials for virus assay.—Animals were exsanguinated by cardiac puncture under ether or chloroform anesthesia. Individual organs designated for separate testing were removed aseptically with separate instruments. Tissues were weighed, homogenized in a mortar or Ten Broeck tissue grinder, and made into 10 percent suspensions in Eagle's basal medium (*4*). The suspensions were clarified by centrifugation at 2500 rpm for 15 minutes in a refrigerated centrifuge. Generally, tissues were assayed for virus on the day of harvest; otherwise they were stored at −60 or −20° C., usually as whole organs, until tested.

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[1] Presented at a Symposium on Phenomena of the Tumor Viruses, New York, N.Y., March 25 and 26, 1960.

[2] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

Urines and mouth swabs were collected antemortem. In the initial experiments, a single voided specimen of urine was diluted in 5 ml. of Eagle's medium; after the importance of urinary excretion had been found, precise dilutions of urine were obtained by collecting the urine in a weighed watch glass, reweighing on a scale with a sensitivity of 5 mg., and rinsing out the glass with an appropriate volume of Eagle's medium. Mouth swabs were taken by twisting a cotton-tipped applicator stick in the mouth; the swab was then squeezed out in a vial containing 5 ml. of Eagle's medium.

Infectivity titrations.—With the exception of one experiment, noted in the text, all assays for infectivity were made by the mouse antibody production (MAP) procedure (*1*). Serial tenfold dilutions of test material were made in Eagle's medium, and were inoculated intraperitoneally into groups of weanling mice of the general purpose stock in volumes of 0.2 ml. per mouse. In routine titrations, 3 mice were used per serial dilution. The mice were tested individually for development of HI antibody response 35 days after injection. Control mice, consisting of uninoculated and saline-injected mice, some of which were placed in cage contact with test mice, were incorporated into each MAP test. In the tests reported here, a total of 1,082 control mice were used, with serologic conversion in 10, which represented a false positive rate of 1 percent.

Titers of tissue-culture fluids and hamster tissue suspensions were calculated by the Reed-Muench method (*5*) and are expressed as the number of 50 percent infectious doses (ID50) per 0.2 ml. of 10 percent tissue suspension. In tests of mouse tissues, harvested after the development of high-titer antibody, titrations were erratic, as described in the text, and the Reed-Muench formula was not applicable. Consequently, titers in the mouse experiments are given as the highest dilution of test material which induced antibody response in more than half the recipient animals.

Hemagglutination (HA) test.—Tissue suspensions were tested for ability to hemagglutinate guinea-pig erythrocytes, with the use of standard procedures described previously (*1*, *6*). In order to "unmask" hemagglutinin from nonspecific inhibitors in tissue extracts (*7*), the tissue suspensions were treated with receptor-destroying enzyme (RDE) prior to testing. This treatment was carried out by mixing equal volumes of tissue suspension and undiluted RDE preparation consisting of unpurified filtrate of *Vibrio cholerae* broth cultures; after incubation at 37° C. for 1 hour, the mixture was heated at 56° C. for 30 minutes, and diluted to a final concentration of 1 percent tissue with *p*H 7.2 phosphate-buffered saline.

Serologic tests.—HI and complement-fixation (CF) tests were carried out, as described previously (*2*, *8*), with antigens prepared with the LID-1 strain. Virus neutralization tests in suckling hamsters were carried out by the following procedure: Tissue-culture fluid infected with the LID-1 strain was diluted 1:5 and mixed with an equal volume of tenfold serum dilutions; the serums had been previously heated at 56° C. for 30 minutes. Virus controls consisted of virus mixed with saline or control mouse serum. The mixtures were held at room temperature for 30 minutes, and 0.2 ml. volumes of each test mixture were inoculated subcutaneously into 1 or 2 litters of 2- to 4-day-old hamsters. Animals dying after the 14th day and all animals surviving at 20 days were autopsied and examined macroscopically for tumors. Neutralization titers of serums were calculated by the Reed-Muench method, calculating the dilution of serum which would reduce tumor incidence by 50 percentage units; for example, since, in the neutralization test on the serums in the first experiment listed in table 9, the virus controls gave 74 percent tumor response (20/27 animals), the titers were calculated as the dilution of serum which would reduce tumor incidence to 24 percent.

RESULTS

Growth of Virus After Inoculation of Newborn Mice With High Dosage

To determine the growth pattern of virus under the conditions usually employed to obtain tumor response—inoculation of newborn mice with high dosage of virus—two chief experiments were carried out. Mice less than 12 hours old were inoculated subcutaneously with 0.1 or 0.2 ml. of a 1:10 dilution of infectious mouse embryo tissue-culture fluid, representing approximately 10^6 mouse ID50 per mouse. The inoculum in experiment 1 was the fifth tissue-culture passage of the LID-1 strain, and in experiment 2 was the seventh passage of the same strain. The tumor response obtained was markedly different between the two experiments: By 150 days after inoculation, 48 of 54 surviving mice in experiment 1 had developed parotid tumors, while none of 21 mice in experiment 2 developed grossly visible tumors by the 150th day, and of 9 mice held for 11 months, only 2 developed tumors. The reason for this difference in response is not known.

Tables 1 and 2 present the infectivity and HA titers of selected tissues obtained at various intervals after inoculation. High infectivity titers were attained by the 7th to 9th day after inoculation, and tended to remain at comparable or slightly lower levels through the 20th to 30th day. Thereafter the titers were lower and more variable.

During the initial peak phase, virus was widely distributed throughout the viscera, and the titers were sufficiently higher than that expected by their content of blood to indicate that the virus was propagating in each organ tested. Highest titers were observed in the kidneys, salivary glands, liver, lungs, and spleen, with somewhat lower titers in thymus, brain, and blood.

After the 2d month of infection, there was a marked difference between the two experiments with respect to the titer of virus detected. The mice in experiment 1 regularly contained virus in the salivary glands, with titers of 10^2 to 10^4, while the mice in experiment 2 were less often

TABLE 1.—Growth of polyoma virus in various organs after inoculation into newborn Swiss mice: experiment 1

Day of harvest	Group	Mice tested	Presence of tumors†	Infectivity and hemagglutin titers* of tissue extracts																					
							Pooled viscera‡																		
				Serum	Blood clot		Including salivary glands		Excluding salivary glands	Salivary glands		Kidneys		Thymus		Lungs		Liver		Spleen		Brain		Urine	Mouth swab
				Virus	Virus	HA	Virus	HA	Virus	Virus	HA	Virus	HA	Virus	HA	Virus	HA	Virus	HA	Virus	HA	Virus	HA	Virus	Virus
2	1	Group of 4 mice	All−				10^4	<10																	
4	2	"	All−				10^5	<10																	
7	3	"	All−				10^7	160																	
7	4	"	All−	10^5	10^4	<20				10^7	640	10^7	5120	10^5	80	10^6	<10	10^7	320	10^6	640	10^5	<10		
7	5	"	All−	10^5	10^5	<20				$\geq 10^7$	640	10^8	5120	10^6	160	10^7	640	10^7	640	10^7	640	10^5	<10		
10	6	"	All−				10^7	<40																	
10	7	"	All−	10^5	10^6	<10				10^6	<10	10^7	5120	10^5	<20	10^7	(40)	10^7	<10	10^7	<20	10^5	<10		
14	8	"	All−	10^4			$\geq 10^7$	<10																	
20	9	"	All−				10^4	<20																	
20	10	"	All−		Trace	<10				10^3	<10	10^6	<10	10^4	<10	10^4	<10	10^3	<20	10^2	<10	10^2	<10	10^2	10^1
30	11	"	All−				10^6	<10																	
30	12	"	All−		Trace	<10				10^5	<10	10^6	<10	10^5	<10	10^5	<10	10^6	<10	10^4	<10	10^4	<10	10^4	10^4
60	13	"	All−				10^5	<80																10^4	10^3
90	14	"	All−				10^0																	Trace	Trace
90	15	"	All+						10^2	10^4														10^2	$<10^0$
120	16	"	All−						Trace	10^2														10^0	$<10^0$
120	17	"	All+						Trace	10^3														10^2	10^0
120	18	"	All+	$<10^0$						10^3		10^2		10^2		Trace		Trace		$<10^0$		10^1		10^0	$<10^0$
133	19	1 Mouse	+						$<10^3$	10^3															
144	20	"	+							10^1															
144	21	"	+							Positive §															

*Infectivity and HA titers of serum, blood clot, and tissues are expressed per 0.2 ml. of 10 percent suspension. Infectivity titers of urine and mouth swab are expressed per 0.2 ml. of a 5 ml. volume of saline solution in which was collected a single voided specimen of urine or in which a mouth swab was rinsed. HA titers determined on suspensions after treatment with RDE.

†Presence of tumors as determined by gross examination.

‡Pool of kidneys, adrenals, thymus, lungs, liver, spleen, pancreas, mesenteric node, gonads, and brain.

§Titer could not be evaluated. *See* table 5.

TABLE 2.—Growth of polyoma virus in various organs after inoculation into newborn Swiss mice: experiment 2

Time after inoculation (*days*)	Mice tested	Presence of tumors‡	Serum HI antibody§	Infectivity and HA titers* of tissue extracts												
				Pooled organs†		Parotid salivary gland		Submaxillary salivary gland		Kidney		Lungs		Liver		Urine
				Virus	HA	Virus	HA	Virus	HA	Virus	HA	Virus	HA	Virus	HA	Virus
5	Pool of 4	All—	<100	10^5	<10											10^1
9	Pool of 4	All—	<100	10^7	160											10^3
9	Individual	—				10^6	<200	10^7	160	10^5	<20	10^3	<10	10^5	<10	10^1
9	Individual	—				10^6	400	10^5	<20	10^7	2560	10^4	<20	10^6	<10	10^3
14	Pool of 4	All—	800	10^5	<10											10^2
21	Pool of 4	All—	1600	10^2	<10											10^4
21	Individual	—				10^6	<200	10^6	<200	10^6	320	10^2	<20	10^5	<20	
21	Individual	—				10^7	<200	10^6	<200	10^8	25, 600	10^6	<10	10^6	<20	
35	Pool of 4	All—	3200	10^3	<10											$<10^1$
65	Pool of 4	All—	1600	10^0												$<10^0$
65	Individual	—				10^1		10^0		10^2		10^0		Trace		$<10^1$
65	Individual	—				$<10^1$		10^0		10^1		10^1		$<10^0$		$<10^1$
90	Pool of 4	All—	51, 200	Trace												$<10^0$
90	Individual	—	6400			$<10^0$		$<10^0$		Trace		$<10^0$		$<10^0$		$<10^1$
90	Individual	—	1600			Trace		Trace		$<10^0$		$<10^0$		$<10^0$		$<10^1$
150	Individual	—	3200			10^1		Trace		Trace		Trace		$<10^0$		$<10^1$
150	Individual	—	1600			$<10^1$		$<10^1$		$<10^0$		$<10^0$		$<10^0$		$<10^1$
150	Individual	—	3200			10^2		$<10^1$		$<10^1$		Trace		$<10^0$		Trace

*Infectivity and HA titers of tissue suspensions expressed per 0.2 ml. of 10 percent suspension. Infectivity titers of urine expressed per 0.2 ml. of a 5 ml. volume of saline solution in which was collected a single voided specimen of urine. HA titers determined on suspensions after treatment with RDE.

†Pool of parotid and submaxillary salivary glands, kidneys, adrenals, thymus, lungs, liver, spleen, pancreas, mesenteric node, gonads, and brain.

‡Presence of tumors as determined by gross examination.

§Pooled serums of mice in the group.

positive at the level of testing, and the titers in the positive mice were very low.

As expected from the high titers of virus in salivary glands and kidneys, the mouth swabs and urine specimens were frequently infectious and occasionally in high titer. More extensive data on excretion of virus have been presented elsewhere (*3*).

Table 3 presents results of a similar experiment, which was carried out to compare the early phase of growth in an inbred mouse strain with that previously described in Swiss mice. Pregnant DBA/2Lw[3] mice were tested for HI antibody, and the litters of antibody-negative mice were inoculated when less than 12 hours old. The inoculum was $10^{6.7}$ mouse ID50 of fifth mouse embryo tissue-culture passage of the LID–1 strain, given subcutaneously. The tissue suspensions were assayed by HA tests. The results were comparable to those in experiments 1 and 2, with high HA titers on the 8th day, and maximal HA titers in the kidneys. It is noteworthy that 4 of the urine samples gave HA at a 1:100 dilution. While these specimens were not tested for specificity of the HA by HI tests, these reactions probably represented specific viral HA, since tests of normal urine have not given positive results at lower dilutions, and in other experiments high titers of hemagglutinin have been found in infected mouse urine, with specific inhibition by polyoma antiserum.

TABLE 3.—Hemagglutinin titers in tissues of DBA mice inoculated with polyoma virus as newborns

Time after inoculation (*days*)	Mice tested	Hemagglutinin titer*					
		Pooled organs	Salivary glands	Kidney	Thymus	Liver	Urine
5	Pool of 3	200					<100
8	Individual		<100	5120	<100	400	<100
8	Individual		400	5120		<100	100
8	Individual		400	2560	100	<100	100
12	Individual		<100	<100	<100	<100	100
14	Individual		<100	<100	<100	<100	100

*Titer per 0.2 ml. of 10 percent tissue suspension, and per 0.2 ml. of urine. All titers determined after RDE treatment.

HA Inhibitors in Serum and Tissue Extracts

The data on recovery of infectious virus must be interpreted in relation to the amount of antibody in the test material. Table 4 shows results of HI tests on the serums and tissue extracts from experiment 1, and table 2, column 4, shows HI tests on serums from experiment 2. To evaluate the content of nonspecific inhibitors as well as of antibody, the tests on tissue extracts were made on the untreated material and on the same suspension after treatment with RDE; the inhibition of HA by the untreated suspension represents the effect of both inhibitor and antibody, while inhibition by the RDE-treated material represents anti-

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[3] Kindly supplied by Dr. Lloyd W. Law.

TABLE 4.—HA inhibitors in tissue suspensions obtained at intervals after inoculation of newborns: experiment 1

		HI titers*																		
			Blood clot		Pooled viscera		Salivaryglands		Kidneys		Thymus		Lungs		Liver		Spleen		Brain	
Day of harvest	Group	Serum	Un-treated	RDE-treated	Un-treated	RDE-treated	Un-treated	RDE-treated	Un-treated	RDE-treated	Un-treated	RDE-treated	Un-treated	RDE-treated	Un-treated	RDE-treated	Un-treated	RDE-treated	Un-treated	RDE-treated
2	1	<100 (pool)			160	<20														
4	2	<100 (pool)			20	20														
7	3	<100 (pool)			NT†	NT														
7	4	<100 (pool)	<20	(20)			>320	NT	NT	NT	NT	NT	<10	<20	<10	NT	<10	NT	<10	<10
7	5	<100 (pool)	<20	<20			NT	NT	NT	NT	NT	NT	20	NT	<10	NT	<10	NT	(10)	(10)
10	6	50; 200; 200; 400			<80	<20														
10	7	200; 200; 400; 3200	40	40			≥1280	<20	NT	NT	<10	<10	<10	NT	<20	<20	<10	<20	40	<10
14	8	200; 200; 1600			320	320														
20	9	3200 (pool)			160	160														
20	10	800; 1600; 1600; 3200	≥1280	1280			≥1280	40	160	80	<20	<20	320	320	80	80	<20	<20	40	40
30	11	6400 (pool)			640	320														
30	12	3200; 3200; 6400; 6400	≥1280	≥1280			≥1280	≥1280	640	640	320	<40	640	640	≥1280	≥1280	160	320	80	80
60	13	6400; 6400; 6400; 12,800			≥1280	≧1280														
90	14	3200; 6400; 6400; 6400			1280	1280														
90	15	3200; 6400; 6400; 25,600			5120	2560	5120	1280												
120	16				640	640	2560	1280												
120	17				320	160	640	320												
120	18	3200; 6400; 12,800; 12,800					640	1280	1280	2560	640	2560	2560	≥1, 240	1280	2560	320	640	160	640
133	19	25,600																		
144	20	51,200						800												
144	21	25,600						1600												

*HI titer of serum expressed per 0.2 ml. of serum; titers of all other tissues expressed per 0.2 ml. of 10 percent suspension.

†NT = No test because of HA by the tissue suspension.

TABLE 5.—Representative titrations of parotid tumor suspensions by MAP and tissue-culture tests

Parotid tumor	HI antibody titers*		Result of assay of serial dilutions of tumor extract by MAP or tissue-culture tests †							
	Serum	Tumor extract	Test	10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Expt. 1, group 15	3200–25,600	1280	MAP	1/2‡	0/3	2/3	3/3	3/3	1/3	
Expt. 1, group 15			MAP	2/2	2/3	3/3	1/3	2/3	1/3	0/3
Expt. 1, group 18	3200–12,800	1280	MAP	0/3	2/3	3/3	2/3			
Expt. 1, group 18			MAP	3/3	2/2	2/2	2/3	0/3	0/3	
Expt. 1, group 20	51,200	800	MAP	2/3	2/3	1/3	1/3	0/3		
Expt. 1, group 20			ME TC	0/3§	2/3	1/3	1/2	0/2	0/3	
Expt. 1, group 21	25,600	1600	MAP	0/3	1/3	1/2	1/3	0/3	1/3	
Expt. 1, group 21			ME TC	0/2	1/3	2/3	1/3	0/2	0/3	

*Titers expressed per 0.2 ml. of serum and per 0.2 ml. of RDE-treated 10 percent tumor suspension.

† Results of 35-day MAP tests and mouse embryo tissue cultures observed for 30 days.

‡ Numerator = number of mice developing HI antibody. Denominator = number of mice inoculated.

§ Numerator = number of tissue-culture tubes demonstrating cytopathic effects and/or hemagglutinin. Denominator = number of tubes observed.

body alone. During the peak phase of virus growth, HI tests were often not possible because of hemagglutinin in the tissue suspensions, particularly those treated with RDE. Antibody appeared in serum, at the level of testing, on the 10th day of infection, and rose progressively through the 20th to 30th day, generally reaching titers of 1:1600 to 1:6400. Antibody in tissues was readily demonstrable after the 14th day, with titers in general agreement with that expected by their content of blood.

Nonspecific inhibitor was found in salivary glands in high titer, which was anticipated from the mucoprotein nature of the erythrocyte receptors necessary for polyoma HA (*6*).

Detection and quantitation of virus in tissues taken late in infection were hampered by the high level of antibody in the tissue suspension. Whereas titrations of tissue extracts taken early in infection gave sharp titration endpoints in the MAP test, the late suspensions gave irregular antibody response over a wide dilution range. Occasionally parotid tumor suspensions gave prozones in MAP or tissue-culture titrations, presumably due to reactivation by dilution. Table 5 shows results of representative titrations of parotid tumor suspensions illustrating these points.

It should be noted that in other experiments we have twice encountered parotid tumor suspensions which gave no evidence of infectivity when titered in MAP and mouse embryo tissue-culture tests, but which yielded specific hemagglutinin and virus when the tumor tissue was grown in explant type culture in roller tubes.

Relationship of Infectivity Titers to Number of Viral Particles in Tissue Extracts

Previous studies (*9*, *10*) have indicated that HA titers of tissue-culture fluids can be used to estimate the concentration of total viral particles, there being approximately a 1 : 1 ratio between physical particles and erythrocytes at the endpoint of an HA titration. The number of particles determined in this manner must be regarded as a minimum estimate, and dependent on the efficiency of removal of hemagglutination inhibitors from the test material.

Since the infectivity titrations on experiments 1 and 2 were not done with sufficient precision to justify calculation of particle-infectivity ratios from the data in tables 1 and 2, it is worthwhile to present data from another experiment carried out under similar conditions in which significant comparisons could be made.

Newborn Swiss mice were inoculated subcutaneously with 0.05 ml. of undiluted infectious tissue-culture fluid, representing approximately 10^6 ID50 per mouse. Seven days later, 1 mouse from each of 5 litters was sacrificed and the kidneys harvested individually; the remaining mice were sacrificed, and the kidneys removed and pooled. The individual and pooled kidneys were made into 10 percent suspensions in

Eagle's medium, clarified, and titrated for infectivity by the MAP procedure; 5 mice per titration dilution were used. Two titration tests were done: the different kidneys of the individual mice being tested in separate tests and the pooled kidney suspension being titrated both times. HA titers were determined on the suspensions after RDE treatment, with 10^7 erythrocytes per HA tube; the estimate of particle number was taken as 10^7 times the HA titer. The results are given in table 6. The ratios obtained varied from $10^{1.6}$ to $10^{3.2}$ particles per ID50, with a mean of $10^{2.15}$. This ratio is comparable to that generally found with polyoma-infected tissue-culture fluids (*10*).

TABLE 6.—Relationship of infectivity and HA titers of mouse kidney suspensions

Mouse No.	Infectivity titer* Right kidney	Infectivity titer* Left kidney	HA titer* Right kidney	HA titer* Left kidney	Ratio $\frac{\text{HA} \times 10^7}{\text{ID50}}$ Right kidney	Ratio $\frac{\text{HA} \times 10^7}{\text{ID50}}$ Left kidney
1	$10^{7.8}$	$10^{8.5}$	800	1600	$10^{2.1}$	$10^{1.7}$
2	$10^{7.6}$	$10^{8.0}$	1600	1600	$10^{2.6}$	$10^{2.2}$
3	$10^{7.4}$	$10^{7.8}$	400	400	$10^{2.2}$	$10^{1.8}$
4	$10^{7.4}$	$10^{6.8}$	100	100	$10^{1.6}$	$10^{2.2}$
5	$10^{5.8}$	$10^{7.0}$	100	100	$10^{3.2}$	$10^{2.0}$
Pool	$10^{8.0}$, $10^{7.9}$		800, 800		$10^{2.0}$	

*Titers expressed per 0.2 ml. of 10 percent suspension.

Development of Hemagglutinin in Mouse Tissues After Infection With Low Virus Dosage

Since the results described in the preceding sections were obtained by inoculation with massive doses of virus, it appeared desirable to determine if a similar growth pattern was induced by low-dose inoculums. Newborn general purpose mice were inoculated subcutaneously with 0.05 ml. of a 10^{-6} dilution of fluid from mouse embryo tissue cultures infected

TABLE 7.—HA titers of salivary-gland and kidney suspensions at intervals after inoculation of newborn Swiss mice with a low dose of polyoma virus

Time (*days*)	Group No. (*4 mice each*)	HA titer* Salivary gland	HA titer* Kidney
10	1	<10	1280
10	2	<10	1280
12	3	<10	80
12	4	<10	5120
15	5	<10	<10
15	6	<10	80
18	7	<10	<10
18	8	<10	<10
21	9	<10	<10
21	10	<10	<10

*Titers expressed per 0.2 ml. of 10 percent suspension.

with a strain of polyoma virus recovered from the viscera of a normal antibody-positive, AKR mouse from the colony of Dr. Ludwik Gross. This fluid had an infectivity titer of $10^{7.5}$ TC ID50 per 0.2 ml. when assayed in mouse embryo tissue culture, and $10^{8.2}$ mouse ID50 per 0.2 ml. when titrated by the MAP procedure; thus, each newborn mouse received approximately 10 to 40 infectious doses of virus. At intervals 2 groups of 4 mice each were sacrificed, and HA titers were determined on the pooled salivary glands and on the pooled kidneys (table 7). No hemagglutinin was detected in the salivary-gland extracts, but the kidney extracts gave titers comparable to those obtained with large inoculums, and the time course of hemagglutinin development and disappearance was similar to that in the previous groups.

Growth of Virus in Weanling Mice

Although weanling mice are refractory to the tumor-inducing effect of polyoma virus infection, they are quite susceptible to infection *per se*, as evidenced by the uniform development of antibody after parenteral or intranasal inoculation of low doses of virus (*1*, *3*). To characterize the pattern of infection in weanling mice, experiments comparable to those described were conducted. Swiss mice were infected when 3 to 4 weeks old by intraperitoneal injection of 0.2 ml. of undiluted tissue-culture fluid containing $10^{6.5}$ mouse infectious doses of virus. Titers of selected organs were determined at various times after infection (table 8).

TABLE 8.—Growth and excretion of polyoma virus after intraperitoneal inoculation into weanling Swiss mice. Inoculum: $10^{7.0}$ mouse ID50

			Virus titer*				
Time after inoculation (*days*)	Mouse No.	Serum HI antibody	Pooled organs, less salivary glands and kidneys	Salivary glands	Mouth swab	Kidney	Urine
8	1	400					
8	2	400					
8	3	200					
9	4	400	10^4	10^4	10^1	10^2	$<10^2$
9	5	400	10^2	10^3	$<10^0$	10^3	$<10^2$
9	6	400	10^3	10^3	10^0	10^3	10^2
12	7	800	10^3	10^3	$<10^0$	10^1	10^4
12	8	800	10^3	10^3	$<10^0$	10^1	$<10^2$
12	9	200	10^3	10^3	Trace	10^1	$<10^2$
21	10	3200	10^2	10^3	10^2	10^2	10^3
21	11	3200	10^2	10^2	Trace	10^1	10^4
21	12	3200	10^0	10^2	$<10^0$	10^0	10^1
49	13	6400	$<10^0$	10^0	$<10^0$	$<10^0$	$<10^1$
49	14	6400	$<10^0$	10^2	Trace	$<10^0$	$<10^2$
49	15	3200	Trace	10^1	10^0	$<10^0$	$<10^2$
210	16	6400	$<10^0$	$<10^0$	$<10^0$	$<10^0$	$<10^2$
210	17	6400	Trace	Trace	$<10^0$	$<10^0$	$<10^1$
210	18	3200	$<10^0$	Trace	$<10^0$	$<10^0$	$<10^2$

*Titers of tissue suspensions expressed per 0.2 ml. of 10 percent suspension; titers of urine expressed per 0.2 ml. of urine.

The growth pattern roughly paralleled that in newborn mice, but the maximal titers obtained were 3 to 4 logs lower, and antibody development appeared to be somewhat more prompt. The tissue extracts in table 8 were also tested for hemagglutinin, and all were negative at a 1:20 dilution. Despite the reduced titers of virus in the tissue suspensions, virus was occasionally present in urine and saliva, in the former occasionally in a relatively large amount. In 2 mice the titer of virus recovered from urine was 100-fold greater than from an equivalent mass of kidney. This could perhaps be due to the greater inhibiting effect of antibody in the tissue suspension or to propagation of virus in the lower urinary tract. As in the newborn mice, virus persisted at a low level in the tissues for prolonged periods, particularly in the salivary glands.

Growth Curve of Virus in Suckling Hamsters

Three experiments were carried out to determine the growth curve of polyoma virus after subcutaneous inoculation into suckling hamsters (*11*). Groups of suckling hamsters 2 to 4 days of age were inoculated with 0.1 ml. of undiluted infectious tissue-culture fluid given subcutaneously on the back. At prescribed intervals, groups of 3 to 6 animals, each from a separate litter, were sacrified, the organs examined in the gross for the characteristic hemangiomatous and sarcomatous tumors, and various organs removed for testing. In some instances, individual organs were tested; in others, a pool was made of the heart, lungs, liver, and kidneys. Each suspension represented corresponding tissues of a number of animals. In experiment #H-1 the virus was assayed by titration in mouse embryo tissue cultures, and in the other two experiments, the titrations were done by the MAP procedure.

Table 9 presents the results of these 3 experiments, giving the course of viral growth, antibody response, and the time of appearance of grossly visible tumors. The inoculums used in the experiments were highly pathogenic; some animals demonstrated grossly visible tumors 10 to 14 days after inoculation, and all animals killed 21 days or more after inoculation demonstrated multiple tumors. The tests for infectious virus titer indicated that a peak of infectivity was reached within 3 to 6 days after inoculation, with highest titers in the kidneys, heart, and liver. After the peak of infectivity, virus titer declined rapidly. In experiment #H-2, the titer in most organs was very low by 7 to 10 days, and, in both experiments #H-1 and #H-2, the pooled organs were generally noninfectious after the end of the 2d week of infection.

Antibody response in the suckling hamsters was prompt and of high titer. HI antibody appeared within 7 to 10 days after inoculation, and neutralizing antibody was also present by the 10th day. Complement-fixing antibody appeared slightly later, at 10 to 14 days.

Additional Attempts To Recover Virus From Hamster Tumors

The data described in the preceding section indicated that tumors removed 3 weeks or later after inoculation were generally noninfectious. Since the previous tests were done with pools of tissues from several animals, additional tests were made with tumors from individual hamsters. Tumorous organs from hamsters sacrificed 16 to 82 days after inoculation were made to 10 percent suspensions in Eagle's medium and tested for infectivity by the MAP and/or mouse embryo tissue-culture procedures. The results are summarized in table 10. Three of the 4 tumors removed at 16 to 17 days contained detectable viral infectivity; however, the quantity was small and required, in one case, blind passage in tissue culture to produce cytopathic changes, and, in the other 2 cases, relatively prolonged observation periods. Of the tumors removed at 43 days, only 1 of the 5 contained detectable virus, and in this instance only in very low titer. The 5 tumors tested at 82 days were negative for virus in both tests, and the tumor extracts did not produce tumors or HI antibody when inoculated into suckling hamsters.

Serial Passage of Polyoma Virus in Suckling Hamsters

The titers of virus obtained at the peak of virus activity in the growth curve experiments were not sufficiently high to establish unequivocably that viral multiplication had occurred in the hamsters. To establish this point, two serial passage lines of polyoma virus were carried out in suckling hamsters. Line 1 was initiated with the pooled-organ suspension taken at 7 days in experiment #H-1 (table 9), and passage line 2 was initiated with the 3-day pooled organs in experiment #H-2 (table 9). Serial passages were made at 5- to 7-day intervals by preparing suspensions of the heart, lungs, liver, and kidneys of 4 suckling hamsters that had been inoculated as 2- to 4-day-old animals, and the undiluted clarified suspension was inoculated into 2 litters of hamsters. The hamsters which were not sacrificed for passage were maintained 5 weeks for observation of tumor incidence. Many such hamsters were also bled at the time of sacrifice to determine if there was an antibody response to the inoculum. Each suspension was also inoculated into 5 weanling mice as a parallel test for viral infectivity. The 2 lines were successfully carried through 19 and 16 serial passages, respectively (table 11). Tumors were observed sporadically throughout the passage series, but were not observed after the virus had been lost. The low incidence of tumors indicated that the virus was not becoming highly adapted to be oncogenic for the hamster, at least in the dosages used in the form of hamster tissue suspensions. The suckling hamster suspension at the 11th passage level was titrated by the MAP procedure and yielded a titer of $10^{5.1}$ ID50 per 0.2 ml., which is comparable to those obtained in the growth curve experiments. This suspension was also inoculated into mouse embryo tissue culture, and virus was recovered; the tissue-culture fluid when inoculated undiluted into suckling hamsters was highly oncogenic, and produced tumors in each of the 11 surviving hamsters within a period of 35 days.

Table 9.—Development of virus and antibody after subcutaneous inoculation of suckling hamsters with polyoma virus

		Macroscopic tumors		Antibody in serum			Virus titer: ID50 per 0.2 ml. of a 10 percent suspension											
Expt. No	Day of sacrifice	No. of hamsters with tumors / No. sacrificed	Types of tumors	CF	HI	Hamster tumor neutralization	Pooled organs	Serum	Heart	Liver	Lungs	Kidney	Gonads	Lymph nodes	Muscle	Brain	Spleen	Salivary glands
H-1	3	0/4					$10^{3.3}$											
	6	0/4					$10^{4.3}$											
	10	2/4	Liver and kidney hemangiomata	<10	2560	200	$10^{2.8}$											
	14	4/4	Hemangiomata of liver, intestines, kidney, lung, bladder, and uterus	160	10240	1000	$10^{1.3}$											
	21	3/3	Hemangiomata of liver and lungs; sarcomata of heart and kidney				$<10^{0.3}$											
	28	3/3	Same	1280	10240	1300	$<10^{0.3}$											
H-2	3	0/3		<10	<40	<100	$10^{4.5}$	$<10^{0.3}$										
	7	0/3		All <10	400 (pool)	<100		$<10^{0.3}$	$10^{1.0}$	$10^{0.8}$	$<10^{1.0}$	$10^{0.5}$	$<10^{0.1}$	$10^{0.5}$	$<10^{0.3}$	$<10^{0.3}$		
	10	0/3		<10, >10, >10	3200 (pool)	>100		$<10^{0.3}$	$10^{0.5}$	$10^{1.1}$	$10^{0.5}$	$10^{2.5}$	$<10^{0.1}$	$10^{0.8}$	$10^{0.8}$	$<10^{0.3}$	$10^{1.1}$	
	15	1/3	Hemangiomata of liver	All >10	1600, 1600, 1600		10^{0}	$<10^{0.3}$										
	21	3/3	Hemangiomata of liver and lungs; sarcomata of heart	All >10	800, 800, 800		$<10^{0}$	$<10^{0.3}$										
	28	2/2	Hemangiomata of liver and lungs; sarcomata of heart and kidneys	Both >10	800, 1600		$<10^{0}$											

	35	6/6	Same; subcutaneous sarcomata	800, 800, 800, 1600, 1600, 3200	$<10^0$											
H-3	4	0/5		<100		$<10^0$	$10^{4.2}$	$10^{4.2}$	$10^{3.2}$	$10^{5.0}$	$10^{2.8}$	$10^{3.5}$	$10^{3.0}$	$<10^{1.0}$	$10^{2.0}$	$10^{3.2}$
	4	0/5		<100		$<10^{0.3}$	$10^{4.5}$	$10^{4.2}$	$10^{3.2}$	$10^{4.8}$	$10^{3.5}$	$10^{2.2}$	$10^{2.8}$	$10^{1.5}$	$10^{3.2}$	$10^{2.8}$

Table 10.—Recovery of virus from hamster tumors

		Results of virus assays on tumor extract							
	Removal after inoculation	MAP test*					Mouse embryo tissue culture	Suckling hamsters (*35-day test*)	
Type of tumor	(*day*)	10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^0	Tumors†	HI antibody‡
Hemangiomata of liver and lung	16	2/2					+ (17–24 Days)		
Hemangiomata of liver and lung	16	1/2					+ (17–21 Days)		
Hemangiomata of liver and lung	16	0/2					− (Blind passage)		
Hemangiomata of liver	17						+ (Blind passage)		
Sarcoma of heart	43	0/3	0/3	0/3	0/3	0/3			
Hemangiomata of liver	43	2/3	0/3	0/3	0/3	0/3			
Hemangiomata of liver, sarcoma of heart, and subcutaneous sarcoma	43	0/3	0/3	0/3	0/3	0/3	− (Blind passage)		
Sarcoma of heart	43	0/5	0/3	0/3	0/3	0/3	− (Blind passage)		
Hemangiomata of liver, sarcoma of heart	43	0/6	0/3	0/3	0/3	0/3	− (Blind passage)		
Hemangiomata of liver	82	0/3					− (Blind passage)	0/13	0/13
Sarcoma of heart	82	0/3					− (Blind passage)	0/16	0/16
Sarcoma of uterus	82	0/3					− (Blind passage)	0/14	0/14
Subcutaneous sarcoma	82	0/3					− (Blind passage)	0/7	0/7
Subcutaneous sarcoma	82	0/3					− (Blind passage)	0/6	0/6

*Numerator = number of mice developing antibody (35-day test). Denominator = number of mice inoculated with dilution of tissue extract.
†Numerator = number of hamsters developing macroscopic tumors. Denominator = number surviving 35 days after subcutaneous inoculation with 0.1 ml. of 10 percent tumor extract.
‡Numerator = number of hamsters developing antibody. Denominator = number surviving 35 days.

TABLE 11.—Tumor response and tests for virus in serial passage lines of polyoma virus in suckling hamsters

		Passage line 1			Passage line 2		
		Suckling hamsters		MAP test	Suckling hamsters		MAP test
Passage	Day passed	Tumors*	HI antibody†	HI antibody†	Tumors*	HI antibody†	HI antibody†
2–10	5–7	10/30	7/7	45/45	6/35	6/6	44/44
11–15	5	7/17	11/11	21/21	4/15	13/13	22/23
16	5	2/2		5/5	0/8	8/8	5/5
17	5	0/8	7/8	5/5	0/1	0/1	0/5
18	5	—		4/5	0/1	0/1	0/5
19	5	0/2	0/2	4/5	0/7	0/7	0/5
20	5	0/5	0/5	0/4	0/4	0/3	0/5

*Numerator = number developing gross tumors during 35-day observation period. Denominator = number observed.

†Numerator = number of animals developing HI antibody ($\geq$ 100 titer). Denominator = number tested.

DISCUSSION

The results presented here indicate that the pattern of growth of polyoma virus in mice and hamsters follows that of the majority of viruses of acute illnesses, *i.e.*, maximal growth of virus within a few days or weeks, followed by decline or disappearance of detectable virus. However, the fact that the peak of viral activity significantly precedes the appearance of the overt manifestation of the infection—the tumor—carries many important implications.

In an examination of the mechanism of tumorigenesis by viruses, two basic patterns must be considered. Either the virus infection induces a cellular alteration which is later expressed as tumorous growth independent of persistence of virus in the cells, or unrestrained cellular growth occurs as a reaction to continual activity of virus in the cells. The growth patterns of polyoma virus, particularly that in the hamster, are highly suggestive of the former mechanism and imply that searches for the cellular alteration leading to tumor formation should concentrate on the early period after infection.

It will be important to determine if differences in tumor response of mice under different experimental conditions can be related to differences in time, height, or duration of peak virus titers or to the titer of persistent virus. The experiments described here demonstrated marked differences in tumor response, but the titration data are not adequate to justify any conclusions and can only serve to suggest hypotheses for further testing. Of the mouse experiments reported here, only experiment 1 represented a highly oncogenic system and must form the basis for comparison. The contrast between this experiment and the growth curve in weanling mice was great, both in magnitude of peak virus growth and in titer of persistent virus. However, a correlation of growth pattern with the marked difference in tumor response between experiments 1 and 2, both conducted in newborn mice, is not as apparent; the most noteworthy difference between the growth curves in these two experiments was the titer of virus in the 2d through 5th months, which was lower in the experiment with poor tumor response. This observation, if confirmed, would support the concept that tumor induction is a result of continued presence of virus.

The concept of an early peak of virus growth, with little or no residual infectivity at the time of tumor development, has important implications for epidemiologic and etiologic studies of tumors in relation to viral infection. If this type host-parasite relation is frequent with oncogenic viruses, studies using tumor cases as the reference point are greatly handicapped.

The extent to which antibody in mouse tissue suspensions appears to interfere with the detection of virus also has important implications for searches for tumor viruses in other species as well as in studies of the virus-tumor relationship in polyoma virus infection. Mouse parotid tumors should provide a useful system for evaluating the efficacy of procedures for dissociating virus from antibody in tissues.

To summarize: The course of development of infectious virus, hemagglutinin, and antibody in various tissues has been studied after inoculation of polyoma virus into newborn mice, weanling mice, and suckling hamsters.

In newborn mice, virus propagated in a wide variety of tissues, reached maximal titers 7 to 14 days after inoculation, and gradually declined thereafter. Highest titers were generally attained in the kidneys. Virus was recovered from the tissues, and particularly from the parotid tumors, for at least 4 to 5 months after inoculation. Antibody developed within 10 days after inoculation, and reached high levels by the 30th day; the high titers of antibody interfered with the detection of virus in parotid tumors by either tissue culture or mouse antibody production tests, giving prozones and erratic endpoints in infectivity titrations.

The infection pattern in weanling mice was characterized by lower titers of virus during the peak phase and prolonged persistence of very low quantity of virus.

In suckling hamsters, virus reached peak titers 4 to 6 days after inoculation and declined rapidly thereafter; tissues were generally noninfectious by the 3d or 4th week after inoculation. Tumors harvested during the 3d week of infection generally contained small amounts of virus, but tumors harvested at 6 to 11 weeks were generally noninfectious. Two passage lines of virus were maintained for 16 to 19 passages in suckling hamsters by transfer of organ suspensions harvested 5 to 7 days after inoculation.

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Discussion

Dr. Stanton (National Institutes of Health): I would like to express admiration for this excellent and extensive biologic and epidemiologic survey reported by Rowe and his associates. We have been making a much more modest epidemiologic survey for polyoma HI antibodies in our own mouse colonies with findings much in agreement with some of those reported by Dr. Rowe.

In a small colony in which polyoma parotid tumors were induced by inoculation of newborn mice with positive tissue-culture supernatant fluids, we found that the untreated control mice in the same room had an incidence of positive HI serums of approximately 60 percent. This is to be compared with an incidence of only 19 percent in untreated mice in the larger colony from which these animals were originally derived. In attempting to assess the sources of dissemination of polyoma virus in this larger colony with a 19 percent background incidence in the untreated animals, we have studied some 24 different transplantable tumors which are carried in this area. In approximately one third of these transplanted tumors, the host mice had a significantly higher incidence than the background incidence of positive serology.

Study of these animals before and after transplantation showed that they were usually converted from negative to positive within about 2½ to 3 weeks after transplantation.

The number of animals tested in some of these tumors was rather small because of the difficulty of keeping alive animals with fast-growing tumors. But the need for allowing the tumor to grow 2½ or 3 weeks to assess the possible presence of polyoma infection in transplanted tumors is very important; more significant than the positive serology was the fact that in 7 of these 8 tumors in which we attempted to isolate virus by tissue-culture methods, we have to date obtained polyoma virus from 5 of the growths. In similar studies on 7 of the tumors with lower or no greater than background incidence of polyoma serology, no polyoma virus was isolated by tissue culture.

No attempt has been made to assess the extent of virus excretion by animals carrying these transplanted tumors. We suspect that possibly in the first 2 weeks after this artificial inoculation of young weanlings and before antibodies are formed, they may excrete virus in significant amounts into the environment.

I would like to ask Dr. Rowe if he has yet studied the rate of excretion of polyoma virus by mice inoculated with infected tumor.

Dr. Rowe: I have that written down to ask you. We have made very few tests, and they have been negative but, epidemiologically, it is almost certain that some of these mice must be good excreters.

Dr. Eddy (National Institutes of Health): I was interested in the observation that the 82-day-old animals were negative on tissue-culture isolation. I was wondering how many blind passages you made. I have had much more experience with hamsters than with mice, but it is unusual not to be able to recover the agent from a virus-induced tumor in the hamster and also in mice that I have tested, even if the animals were very old, though the virus is not always found on the first passage. Sometimes two or three blind passages in tissue culture are required, as with influenza or many other viruses. I was wondering about the negative result with the 82-day-old mouse.

Dr. Rowe: We made only one blind passage. We would not dare make more than one because of the great increase in risk of contamination with further passages in a contaminated environment.

Dr. Eddy: A significant matter mentioned in your paper that you did not discuss here is concerned with the importance of controls. Since, from the beginning, we noted that the virus spread over the laboratory quickly, we instituted what we refer to usually as Monday controls. Each lot of new cells is incubated in the usual way for 2 weeks, and on Monday morning before any other virus is handled, the fluid from the cultures that are 2 weeks old is transferred to new lots of mouse embryo cells. These are in addition to all the other normal cultures that we carry with every single virus passage. Thus we are able to be certain of two things: (1) We do not have or have not encountered polyoma virus in the mice that we are using, and (2) we have a check on our technique.

Furthermore, I think that in any work with any mouse colony it is important that the animals are uniformly susceptible, since there is less chance of the presence of a contaminating virus.

Dr. Habel (National Institutes of Health): In view of the ease with which mice carrying other types of tumors pick up polyoma virus, it is interesting that we have a transplantable polyoma virus-free tumor that, as far as we know, was originally induced by polyoma virus by inoculation of the agent into newborn mice. This was done by Dr. Eddy. The fibrosarcoma developing in this mouse at 6 months after inoculation has been carried through ten transplants in our laboratory in which we are working with polyoma virus. It has been consistently negative for the virus, and the host animals have shown no antibody. Thus, here is a virus-free tumor which, presumably, was initially produced by polyoma virus and is being carried in the laboratory in which the polyoma virus is present.

On the other hand, we have a corresponding line of tumor in mice that has been consistently positive for virus through ten transplants. It is interesting here that, occasionally, with Dr. Rowe's indirect method of inoculating an emulsion of this tumor into adult mice and then bleeding them and testing for antibodies, certain passages of this consistently positive tumor will be negative by the MAP test. Yet, when we carry the tumor in tissue culture for 21 to 28 days, in every instance we get cytopathogenic effect (CPE) in the developing cells and the appearance of hemagglutinating virus in the supernatant fluid of the culture, even though the original tumor emulsion might have been negative by the indirect test of inoculating mice. Thus, although it may be laborious and may introduce the possible hazard of tissue-culture contamination, here is another means, as Dr. Eddy mentioned, which, with material carried through several subcultures, brings out virus that may have been masked by the presence of antibodies in the original material.

Dr. Henle (University of Pennsylvania): When my colleagues, Dr. Gertrude Henle and Dr. Philip Garnard became interested in the polyoma virus, the hemagglutination phenomenon associated with the polyoma virus had not yet been recognized. To ordinary virologists, a wait of 2 weeks or longer for the development of CPE in mouse embryonic tissue in culture seemed rather arduous. Consequently, we thought that perhaps one could get evidence of viral multiplication earlier by the interference phenomenon. The data we have obtained in this way are similar to those which Dr. Rubin mentioned earlier.

If 10^5 tissue-culture doses of polyoma virus are injected onto mouse embryo cultures, complete resistance to the virus of vesicular stomatitis (VSV) will become apparent in 3 to 4 days. If a smaller amount such as 10^2 doses is inoculated, 10 or more days may be required until resistance to VSV is complete. Thus one can obtain a rough idea of the concentration of polyoma virus in a given preparation on the basis of the time necessary for the induction of resistance to VSV.

The challenge with VSV can be applied at a time when hemagglutination is not as as yet detectable, but protection is obtained. Nevertheless, by the time the results are available, 2 or 3 days later, there is usually some measurable hemagglutination, so that VSV interference is not of extreme practical importance. Another point of interest is that by the time resistance to VSV is measurable, relatively few cells can be shown to contain specific polyoma antigens when they are stained with fluorescent antibodies.

There has been a rumor that interferon has been demonstrated in polyoma-infected cultures. When I tried to learn the source of it I was told, "Wasn't it observed in your laboratory?" I can put this rumor at rest for the time being. If you believe that interference and interferon production are synonymous, we certainly have shown it, but all our efforts so far to demonstrate interferon in polyoma-infected cultures have failed. This might merely be on a quantitative basis, and further efforts are under way, by concentration procedures and other means, to demonstrate an interferon in these cultures.

Dr. Koprowski (Wistar Institute): There is no one here from Dr. Dulbecco's laboratory to comment briefly on his results in tissue culture. Would you care to do it, Dr. Rubin?

Dr. Rubin (University of California): I can only be brief, because all this work was done after I left. If one makes primary cultures from mouse embryos, one gets largely an outgrowth of fibroblasts. When Dulbecco and Vogt infected these primary outgrowths with polyoma virus, they observed a great deal of cellular destruction. This continues for some time with continuous release of fairly large amounts of virus, but ultimately the amount of virus decreases. At about the same time, a new type of cell appears in the culture which is specifically a much smaller cell with the characteristic that it will grow to about 3 or 4 times the cell concentration that the fibroblast will.

This cell inoculated subcutaneously in the mouse will cause a tumor, and, further, this cell is resistant to superinfection by polyoma virus. More or less the same thing has been done with hamster cells, I believe.

Dr. Sanford (National Institutes of Health): I want to comment on this work of Vogt and Dulbecco in relation to what we have done with the mouse parotid cell. They did not find CPE so much in the hamster cells, and it was one of the points in their article that this was not a selective action of the virus on the cell population to bring out a cell that might already have become neoplastic.

In our work with single-cell clones from the normal parotid we do get this CPE, and the recovered cultures have been implanted into adult mice along with control cultures at rather high concentrations of cells. In the transplantation of infected cells to prove whether they are malignant, autonomously growing cells, it is important to know that the implanted cells are the source of the tumor and that the virus released by the cells has not induced a tumor in the host. Controls can be run of the specific cell-free virus material, but one can never be sure whether filtered virus controls of this sort are going to be quite the same in their reaction on host tissues as cells that are implanted and are continuously releasing active virus into the host tissues.

We would like to suggest a system in which we have implanted C3H cells into the hybrids (C3H × BALB/c)F_1. The tumors induced can then be subinoculated, and all our subinoculated tumors grow progressively in the hybrid and in the C3H mouse, but not in strain BALB/c.

Since the hybrid tissues, especially in this combination, are not ordinarily transplantable to the C3H mouse, it is thus possible to tag genetically the cells that are introduced and to prove that the tumors have actually arisen from the implanted cells.

Dr. Dawe: It is important, in interpreting the results of both Dr. Sanford's work and that of Dulbecco and Vogt to take careful account of the morphology of the tumors that are induced from the fibroblast cultures. I have seen the tumors that Dr. Sanford obtained by transferring infected cultures to mice, and the ones that we have obtained from transplanting infected organ cultures back into mice. Those from our organ cultures are identical with the induced tumors of the parotid gland, whereas those of Dr. Sanford's studies are definitely dissimilar and are, by her interpretation, with which I agree, more similar to the so-called tissue-culture tumors which she has obtained previously from isolated cell lines.

Dr. Dmochowski (M. D. Anderson Hospital): An associate of mine, Miss Elizabeth Bereczki, is engaged in the study of the relationship of polyoma virus to host cells. Among other results I would like to mention those obtained by acridine orange staining of mouse embryo cells infected with polyoma virus. The inclusions which are formed after infection are Feulgen negative. Then, eventually, they become lavender and usually remain so, though they sometimes turn pink. However, they take counterstain with azure blue.

I will not go into an interpretation of this, but it should be mentioned that the inclusions do not correspond to the virus particles seen in the electron microscope, as shown by Miss Bereczki. Thus these inclusions we often see may be an expression of reaction of the host and not the representation of aggregates of the virus itself.

Significance of Ultrastructure in Virus-Induced Tumors [1, 2]

FRANÇOISE HAGUENAU, *Institut de Recherches sur le Cancer, Villejuif (Seine), France, and Collège de France, Paris, France*

THANKS to electron microscopy, the modern cytologist has a clear and beautiful picture of the normal cell. The fine structure of the cell's various organelles has been revealed (*1, 2*), classical controversies have been settled (*3, 4*), and a representation of cellular submicroscopic organization has been defined in a universal manner (*5, 6*). The validity of the morphologic results thus attained has been established. In contrast to the opinions prevalent only 5 years ago, no one will deny this any longer since truly satisfactory methods of tissue preservation have been devised, which can be judged by comparison with phase contrast pictures of living cells.

But the situation is quite different when pathology and cancer, in particular, are concerned. Either no real difference between the ultrastructure of cancerous cells and that of normal cells was observed, or cellular lesions, when present, could not be related with certainty to an etiologic factor (*7–10*).

It is the purpose of this brief survey to re-evaluate, after the passage of many years of extensive search, the worth of ultrastructural morphology as a tool in cancer research.

Virus-induced tumors will be given special consideration because these growths appeared at first ideally fitted to studies with the electron microscope, since cancer is a cellular disease, and viruses could be observed visually for the first time. Yet, despite the remarkable observations of particles in Rous sarcoma cells and in cells of mouse mammary tumors, by Claude, Porter, and Pickels (*11*) and Porter and Thompson (*12*), further demonstration of viruses in viral tumors was long delayed and these early discoveries could not be substantiated for many years.

During these periods of difficulties and struggle, investigators realized the importance of detecting indirect signs of viral involvement in the affected cells, since direct evidence of virus particles was difficult to obtain. The first part of this report will deal with the search for such signs. Another necessity was for the electron microscopist to develop sys-

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[1] Presented at a Symposium on Phenomena of the Tumor Viruses, New York, N.Y., March 25 and 26, 1960.

[2] This work was supported in part by research grant C-4602 to the Institut de Recherches sur le Cancer, Villejuif (Seine), from the National Cancer Institute, National Institutes of Health, Public Health Service.

tems in which virus particles multiply and reach higher intracellular concentrations than under natural conditions. This will be the subject of the second part of the present report.

GENERAL ULTRASTRUCTURE OF VIRUS-INDUCED TUMOR CELLS AS COMPARED WITH THAT OF NORMAL CELLS

The fine structure of the different elements of the normal cell need not be detailed here, since numerous and excellent reviews exist on the subject (*1*, *2*). Some characteristics, which are particularly striking in virus-induced tumor cells, will be discussed.

Nucleus

We would like to draw attention to some features of the nucleus of virus-induced tumor cells that may be related to a viral etiology though unspecific in themselves.

Among these, *coarseness of chromatin networks* associated with patchy destruction of nucleoplasm is extremely frequent. Such features are well illustrated by comparison of the relatively well-preserved nucleus of a human breast cell, figure 1, with the nucleus of a Rous sarcoma cell shown in figure 2. The possibly important meaning of such aspects of the nucleus is difficult to appreciate, as far as virus involvement is concerned, because this picture is also that of a badly preserved nucleus.

The recent observations in the study of the Shope papilloma of the rabbit (*13*) and of the mouse polyoma (*14–17*) must, nevertheless, lead us to revise our former attitude toward such images.

Nuclei in thin sections of rabbit papilloma cells appeared at first sight to be poorly preserved. The nuclear membrane was thickened with remnants of chromatin appended to it, while most of the nucleoplasm was often a clear, empty space. High resolution pictures, however, then showed that some of these remnant threads of chromatin constituted the same particles that had been identified in pure preparations of the virus (*18*). When these small amounts of virus were not there, as in most nuclei, the specificity of the destructive lesion could not be established. Yet a relation to the development of viral particles is almost certain.

The same is valid for polyoma. Nuclei of renal tumors of mice appear damaged to the electron microscopist who is looking for undistorted tissues, yet most of the time no virus is found in these tumors (*19*). It is possible, though, that this appearance of the nuclei is linked with the disease as suggested by the nuclei in cells infected *in vitro*.

Another structural characteristic that raises the same problems is the peculiar distribution of chromatin along the nuclear membrane known as *margination of chromatin*, an arrangement often found in tumors.

It is difficult to believe that this phenomenon, as viewed in the electron microscope, is a specific indication of viral involvement, because it is always accompanied by lytic changes comparable with those due to poor preservation; yet it is a classic and conspicuous lesion in infectious viral diseases, even in well-preserved, osmium-fixed nuclei (*20, 21*). Its value as an indication of direct viral involvement cannot be doubted in this example, since it is accompanied by development of clear areas in the center of the nucleus in which the virus particles will later appear.

An unusual feature sometimes present in the nuclei of tumor cells, virus-induced and otherwise, is also noteworthy, though its significance is unknown. It consists of *areas of peculiar granules* larger in size than the normal nuclear granular constituents (figs. 2 and 3). They are present sometimes in Rous sarcomas and have been well illustrated in a fowl tumor induced by 1,2,5,6-dibenzanthracene (dibenz[*a*,*h*]anthracene) (*22*). A similar observation has also been made in a mouse lymphoma (*23*) and in human breast cancer (personal observation). The appearance of such particulate elements in the electron microscope should be known to the morphologist. They are in the range of size of the smaller viruses, and since no membrane has been detected up to now in some types of polyoma virus particles, it is not completely irrelevant to consider the possibility of confusion, especially where virus particles are few in number. Their resemblance, also, to granules found in the nuclei of cells infected with herpes B virus, which have been described as possible "templates" for the formation of that agent, should be considered (*21*).

As to the nucleolus, its fine structure which the electron microscope has revealed (*24, 25*) is not modified in cancer cells of human origin or in those of virus-induced animal tumors. But hyperplasia brings out some of its features in a striking manner (figs. 4 to 7). This is especially true when the cancer cells are grown in tissue culture, and the thinness and good preservation of the specimens allow a true unrolling of the nucleolar ball. The "nucleolonema" then becomes conspicuous and condensation of some of its segments is revealed, while the "pars amorpha" may accumulate in small pools as if microsecretion had occured (fig. 4).

With the exception of the granules illustrated in figures 2 and 3, all these characteristics of the nucleus—coarseness of chromatin network, margination of chromatin, and hypertrophy of nucleoli—have their counterpart in the optical microscope. Much more impressive, therefore, is the following trait.

Recent studies have brought out one striking character of nucleoli and nucleoplasm of virus-induced tumor cells. It consists of the occurrence of very *dense, deeply stained bodies* distributed at random in the nucleoplasm or in the nucleolus (figs. 3 and 5). These, when seen at high magnification, sometimes consist of granules similar in size to the nucleolar granules. Sometimes they seem opaque. Although such bodies are found outside the nucleolus, in the chromatin network, their relationship to the nucleolus is unquestionable. Dense bodies were observed for the first time in the adenocarcinoma of the leopard frog (*26*) and later in the benign human-skin tumors of molluscum contagiosum (*27, 28*). They may be numerous in Rous sarcoma cells cultivated *in vitro* (*29*). In addition to molluscum contagiosum and Rous sarcoma, "dense bodies" are present also in great quantities in polyoma virus-infected cells *in vitro* (*14*, *15*). What makes them important with respect to our problem here is that they are found also in some "classical" virus infections such as herpes B (*20*), adenovirus (*30*), and varicella (*31*). Although they do occur in normal cells—especially when rapidly growing—they are only scattered and scarce and never present in such an amount as that depicted here. It looks as if the "dense bodies" were capable of becoming hyperplastic under certain conditions, and because of their occurrence in a few virus-infected cells, they may sometimes be truly indicative of viral involvement. In this respect it is important to note that they have been found in human breast cancer (*32*).

To conclude this survey concerning the nucleus, one should also briefly mention the nuclear membrane. This is not altered in tumors, virus-induced or otherwise. General hyperplasia of the nucleus often leads to its profound indentation. In tangential thin sections, the characteristic nuclear pores then become remarkably conspicuous, a feature of which the morphologist in search of virus particles in tumor cells should be well aware (*see* fig. 20).

A recent relevant observation, probably related to the presence of virus, if not to virus tumors, is the remarkable reduplication of the nuclear membrane described in adenovirus-infected HeLa cells by Gregg and Morgan (*33*). But none of these changes may be considered as specific.

Cytoplasm

In the cytoplasm the situation is similar, though a certain number of features indicative of modified metabolism are present with unusual frequency in cancerous cells of virus-induced tumors and of human cancers. They are similar to those found in "classical" virus infections.

Although mitochondria may retain a perfectly normal aspect, they may also often exhibit pathologic features (*34*). "Cloudy swelling" is the most frequent lesion (figs. 8 and 12). Transformation into "microbodies" is also extremely common (*35*). This phenomenon is not necessarily indicative of chondrioma hyperactivity. The view that "microbodies" are precursors of adult mitochondria is no longer held by modern biochemists since the discovery of "lysosomes" (*36*).

Other types of transformation also occur, some of which are undoubtedly of a degenerative nature (figs. 9, 10, and 11). To this category belong large bodies presenting the structure of lipides which have undergone the modification known as "myelin figure."

In "classical" virus infection, such bodies should be well known to electron microscopists. Their dimensions and density meet some of the criteria of an "inclusion body" as observed in the optical microscope, and errors have been made in interpreting them as viral inclusions. Yet they are not related to a viral process but most certainly represent a general cellular reaction to abnormal conditions.

Still another type of structure should be described here, because it is at the heart of our matter. In some virus-induced tumors and in avian myeloblastosis in particular, virus develops within formations called "gray bodies" by Bonar *et al.* (*37*). There is impressive evidence that gray bodies correspond to mitochondrial precursors (*38*).

In avian erythroblastosis (*39*), also, remnants of characteristic double membranes and cristae may be seen at the periphery of similar bodies, and they have been observed recently in Rous sarcoma cells grown *in vitro* (*40*) (*see* fig. 25). Undoubtedly, gray bodies play an important though not yet explained role in virus development. When they are found without viral particles, relationship to viral etiology is difficult to perceive but should be kept in mind.

The other classical constituents of the cytoplasm, ergastoplasm, Golgi apparatus, and endoplasmic reticulum, are not especially modified in virus-induced tumor cells or in human cancer.

As is now well established (*41*), the ergastoplasm comprises in its "organized form" three components: the membrane system, the granules attached to it, and the substance contained between the membranes. Cytoplasmic basophilia is linked to the granular component which represents the ribonucleoprotein as demonstrated by the brilliant work of Palade (*42*) and Siekevitz (*6*).

In many cancers cytoplasmic basophilia and ribonucleic acid (RNA) content increase. It is then not surprising to witness in some tumors an increase of ergastoplasm, especially in the form of scattered ribonucleoprotein granules. Yet no general rules are valid, since this increase may not occur. When it does, it is probably linked to rapid multiplication of a dedifferentiated cell in a state comparable to that of the embryonic cell (*43*). One morphologic fact is certain: A striking increase in ribonucleoprotein granules may sometimes be observed in the vicinity of virus particles or in cells where virus formation is occurring, at least in Rous sarcoma (figs. 13, 14, and 15). A similar observation has been made in the study of the Western strain of equine encephalomyelitis. (Morgan—personal communication.) A relationship between this increase and the development of viral particles is thus strongly suggested, which is in good accord with our knowledge of viral chemical composition.

The Golgi zone (*44*) deserves special attention because its hyperplasia in some cancers was noted a long time ago. As all cytologists know, it corresponds to the cellular center which becomes conspicuous in cultures of some virus-infected cells or in cancer cells grown *in vitro* (*45, 46*). Hyperplasia of the paranuclear zone is beautifully illustrated in Rous sarcoma cells grown in tissue culture (fig. 16) and was even considered by Tenenbaum and Doljanski in their fundamental work on the cytopathology of the Rous sarcoma cells (*47*) as the main change occurring in the cytoplasm.

There is no doubt that, like the giant mitochondrial bodies noted, such areas, because of their often well-demarcated limits, have been believed to be "inclusion bodies." Yet there is no difference between the ultrastructure of this area and that of a normal Golgi zone in a cell in which it is well developed, such as the plasma cell (archoplasm). The "inclusion body"-like effect and demarcation from the rest of the cytoplasm are due to dense packing of the Golgi elements (fig. 17) and often to distention of the Golgi vacuoles by a substance of unknown significance. It is of interest to note, particularly in the Rous sarcoma in which this hypertophy is a marked feature, that no relationship between it and virus formation has been observed.

This consideration is important because a relationship between Golgi apparatus and virus particles has been stressed in some strains of mouse mammary cancers (*48*). There is no doubt in this case that the intracellular particles termed type A by Bernhard (*7*) are concentrated in the Golgi region where they may accumulate in such numbers that an "inclusion body" is visible in the light microscope (*49*). But aside from the presence of particles at this site, the participation of the Golgi apparatus in the formation of such particles is not clear. Furthermore, the meaning of the intracellular type of particle in mouse mammary cancer is subject to much discussion, since it has been shown that this was not the infective particle (*50–52*) and that the latter could develop in some mouse strains without the necessary intermediate occurrence of the A type of particle (*50*).

One should now discuss still another type of particulate formation, which is concerned with the complex of "particle-containing" vacuoles described as "compound vacuoles" by Low and Freeman (*53*). This structure is found in many types of normal cells, often in the Golgi region and, typically, vesicles are always present in the cytoplasm immediately adjacent to the vacuole membrane (fig. 18).

In mouse mammary cancer comparable particle-filled vacuoles are frequent among the type A particles. They are striking in K-virus-infected cells (*54*). Aggregations of comparable vacuoles have been described in Lucké's renal adenocarcinoma (*26*). In human breast cancer, attention has been drawn (*32*) to formations that may resemble this, though the absence of vesicles around the vacuolar membrane or even the disappearance of this membrane casts some doubt as to their similar nature (fig. 19). The regularity sometimes of the particles themselves has even led—on purely morphologic grounds—to discussion of their possible viral nature. Arguments in favor of their being a perfectly regular structure of a normal cell are equally strong. It is important, nevertheless, to be aware of them.

Surface Membrane and Endoplasmic Reticulum

Pseudopod activity or hypervillosity of the cell surface is consistent in tumor cells, whatever their origin. These phenomena represent a cell reaction of general significance and cannot be considered as characteristic when observed in the absence of viral particles. The same may be said of the hyperplasia of the endoplasmic reticulum, which can be marked in some virus-infected cells, especially in those grown *in vitro* (fig. 22). Incredible amounts of small vesicles can often be seen in relation to the membrane surface, and their dilatation into vacuoles can be followed (fig. 23). Whether this is a sign of "micropinocytosis" and corresponds to ingestion of the nutrient fluid medium or whether it bears a relation to viral particles cannot be settled from static pictures.

What then is the value of ultrastructural morphology in cancer research? It will be seen in the second part of this survey that far from being deceptive, electron microscopy has become a fundamental tool. If there have been described cell reactions to which one can attach little or no specific meaning, the discussion, nevertheless, has been by no means without a significant bearing on the problem for the following reasons:

1. This paper was meant to constitute a basis for discussion rather than a review.
2. It is important to describe in detail the structures indicated only to avoid conferring on them interpretations of particular significance.
3. We are able to stress the absolute similarity, as far as ultrastructure is concerned, between virus-induced tumors and human cancers.
4. The one surely distinctive positive sign of cell involvement remaining to the ultrastructural morphologist in the study of tumors is the presence of the virus itself.

Although this last assertion must seem quite "obvious," this is not actually true, as will be appreciated from further consideration.

SIGNIFICANCE AND INTERPRETIVE VALUE OF PRESENCE OF VIRAL PARTICLES IN TUMOR CELLS

Difficulty of Morphologic Detection

In some virus-induced tumors, the morphologic observation of virus particles in the tumor tissue itself presents no difficulty. The Shope fibroma of the rabbit is a good example (*55*). The high infectivity of extracts of the growth is related to the presence of large quantities of virus. The agent belongs to the pox group and is, therefore, of large size (around 200 mμ). The development of the virus particles can be followed *in vivo* as well as *in vitro* as early as the 6th hour after infection (*56*). The same applies, also, to molluscum contagiosum (*27*, *28*, *57*), because this disease, also, is caused by a virus of the pox group, the members of which are all of large size. Titration methods have not been developed for the etiologic agent, but the great quantity of particles may be regarded as related to high infectivity. These two examples fall into a special category of probably exceptional importance because of the link they represent between tumors and classical pox infections.

Apart from these conditions, and with the exception of some strains of mammary cancer, there is no virus-induced neoplasm in which detection of a viral particle with the electron microscope has not meant, at the beginning, a tremendous effort and in which tenacious search has not been necessary. In the Rous sarcoma, Claude, Porter, and Pickels (*11*) succeeded in finding particles immediately, but this could not be repeated for a long time in spite of an arduous search (*58*). When at last particles were detected, their extreme rarity was striking (*59*); only 0.38 percent of the cells contained particles. The scarcity of particles has continued to be remarkable, also, in subsequent studies (*60*, *61*). In the Murray-Begg endothelioma, though virus was found (*62*) in 33 tumors examined, the particles were extremely difficult to detect, and sometimes only 2 or 3 were found after hours of search in many samples. This was also true of Fujinami's sarcoma (*63*) and of the fowl leukemias when the search was made for particles in circulating blood cells (*37*).

In the mouse, intracellular particles may be extremely scarce, as in the leukemic organs of mice with Gross' virus-induced leukemia (*64*) and in some strains of mammary tumors in which no particles were found (*48*), even though the Bittner agent could be demonstrated biologically (*52*, *65*, *66*). Moreover, virus particles were not found at first in mouse polyoma tumors studied *in vivo*. It was only later that these were observed in hamster kidney tumors induced with a strain of polyoma virus isolated in Toronto (*19*). The picture of the Shope papilloma virus observed (*13*) for the first time in skin growths was revealed in only 1 case out of 19 examined (*67*).

Other examples could be found to illustrate the difficulty with which particles are sometimes detected. This needs to be stressed repeatedly, because it is by no means apparent when pictures of various tumor viruses, sometimes spectacular, are being shown. The difficulties in this respect are not only of great practical importance but of much theoretical meaning. Since no other ultrastructural sign is characteristic, the presence of the viral particle in a tumor acquires a fundamental significance, if its morphology, as seen, for example, in purified preparations (fig. 21), and the experimental conditions are well defined. It is thus of critical importance to seek strains of animals in which intracellular virus particles are plentiful. Such may represent laboratory "monstrosities," a criticism sometimes raised, but the essence of caricature is but to emphasize and thus better to reveal some traits of the model. In the study of neoplasms in which particles are not readily detectable for one reason or another, the role of the investigator, and particularly of the electron microscopist engaged with the viral hypothesis of cancer, is to attempt to develop the conditions under which virus particles become morphologically detectable.

It is remarkable that this has really been achieved. One has been able to show that it is possible to modify the quantitative aspects of cell-virus relationships in such a manner that virus particles can be detected in the electron microscope relatively easily where previously they had not been apparent. Studies with Rous sarcoma will serve to illustrate this.

Example of Rous Sarcoma Studied *In Vivo*

The problems raised by this avian neoplasm are precisely those of the problem of cancer in general and were they solved, the knowledge of carcinogenesis in general would have been advanced enormously.

It is to the great credit of Bryan and his collaborators to have obtained relatively purified reproducible preparations of Rous virus (*68*, *69*), a remarkable achievement in itself, since the difficulties of the purification of the virus are extreme (*70–72*), and to have demonstrated, in a series of outstanding experiments, the relationship between the amount of recoverable virus in the tumor and the amount of the agent used as inoculum (*73*, *74*). Tumors produced with small amounts of virus yielded little or no extractable agent, and it could be demonstrated further by electron microscopy that a high level of infectivity of extracts of the tumor was paralleled morphologically by a large number of intracellular viral particles (*61*, *75*). This not only provided the advantage of bringing additional proof that the particles observed in the electron microscope were indeed the Rous etiologic agent but demonstrated that experimental conditions could be devised to yield tumors in which otherwise virus particles could be difficult to detect but which now contained an abundance of the agent. It could be clearly seen that, in growths yielding extracts below a certain infective titer, no particles, or very few of them, could be found in the electron microscope, while a remarkably large number of particles could be demonstrated in tumors from which extracts of high infectivity were obtained. The data in this area of study are being confirmed now on a much larger scale.

From the point of view of human tumors, these observations are of great theoretical importance, since it is a reasonable hypothesis that tumors under "natural" conditions in man probably compare with the low-titer group. At any rate, the experimental findings already described account for the phenomenon of the absence or scarcity of particles in all previous studies of the Rous sarcoma. Thus, the notion of "virus-enriched" preparations for electron microscopy was imposed upon electron microscopists in a realistic way. Such material became available in studies *in vivo* in the Rous sarcoma as a result of the efforts of a team who had devoted themselves during years of research to the complete study of that tumor. But practical drawbacks of this system, the need for great quantities of birds, and especially the insecurity due to the presence in these of 10 percent latent contamination with lymphomatosis virus, need not be emphasized. For these and other reasons, the great advance of the last few years has been the demonstration that a parallel enrichment in the content of tumor viruses in cells may be achieved through tissue culture (*76*). From the special point of view of the electron microscopist the advantages of this method are many, in addition to the increase in particles:

1. Better preservation of the ultrastructure of cells may be obtained.
2. Phenomena may be observed with precise timing after infection, and a cytologic cycle of virus characteristics may become apparent.
3. Biologic tests may be effected in parallel with electron microscopy on samples in which homogeneity of the virus preparation is well assured.

The reason that only few virus-induced tumor systems have been studied up to now in this combination is explained by the difficulty of developing susceptible cell strains, that of finding optimum conditions fo virus multiplication, and the often unsatisfactory degree of purification of tumor viruses.

Five systems have been investigated thus far in tissue culture: the Shope fibroma (*56*), polyoma (*14–17*), mouse mammary cancer (*50, 77*), myeloblastosis (*37, 38*), and the Rous sarcoma (*29, 40, 78*). The fundamental findings with certain of these systems will certainly have been considered in detail in the section of this symposium devoted to tissue culture and will not be dwelt upon here, except for the problem of the Rous sarcoma. It may be recalled, though, before discussion of the details of the work with Rous-virus infected cells, that it was through tissue culture that polyoma virus was observed morphologically for the first time, and that proof could be given, also, for the first time, that the particles observed in mouse mammary cancer in the electron microscope were the causal agent. In myeloblastosis, likewise, the contribution of tissue culture has been enormous. Results in this instance are of prime importance, since they are concerned with a system extensively studied and in which biologic and biochemical tools are at hand as in no other virus-induced tumor (*79–81*).

Example of Rous Sarcoma Studied *In Vitro*

Studies with the Rous sarcoma in tissue culture in correlation with electron microscopy will be dealt with more at length, since they involve our own work (*29, 40, 78*). Here, again, the most important observations are concerned with the agent itself. Whereas the study of the cancerous cells obtained from tumors in the bird revealed particles of only one type (fig. 24), as described by several authors (*60, 61, 82*), the particles found in tissue culture display new aspects. In the tumors from the bird, particles are always extracellular, and even when apparently inside the cell, they are still always extracytoplasmic, contained in vacuoles often communicating with the surface membrane (fig. 23). No absolute proof then can be gathered from these pictures as to the actual site of virus formation. Such images could illustrate engulfment of particles in the external medium as well as their production and release.

After infection of cultures with Rous sarcoma virus, from preparations of supernatant fluids of previous passage tumor-cell cultures, the infective titer, as measured by titration on the chorio-allantoic membrane, reached its highest level at 10 days. Electron microscopy showed an absolutely remarkable increase in this period in the number of virus particles of "classical" type. This increase in particle number can be correlated with the titer by direct counting (*78*).

In addition, two new morphologic aspects of the particles become apparent. Some particles unequivocally intracytoplasmic are revealed (fig. 26). They occur in dense clusters so that the units are not always easily discerned. Each particle possesses a large nucleoid less dense than that of the classical particle. The outer membrane is delicate and surrounded by distinct ribonucleoprotein granules. The mean diameter of the whole particle measures approximately 40 mμ and that of the nucleoid 33 mμ. Since all the viral particles are tightly pressed together with no space between, they appear as if embedded in an agglomeration of ribonucleoprotein granules. Such groups of particles have been found thus far very rarely and only at the height of "classical" virus production. Bernhard, Oberling, and Vigier (*82*) had already observed in some tumors a "granular mass" which they believed to be a kind of "viroplasm" from which typical virus could be formed. Difficulties in the preservation of chicken sarcoma tissue prevented observation of the details seen in tissue culture, yet there is no doubt that these were the same formations. This constitutes, therefore, some evidence in favor of a relationship between this new type of particle and the "classical" Rous virus particle, though the possibility that they are another unrelated virus will have to be ruled out.

The other phenomenon observed in tissue culture was concerned with the "classical" virus particle. Again, at the height of infection, the extracytoplasmic viral particles are very numerous and can be observed not only in typical vacuoles well limited by a membrane, as is the rule, but embedded in a gray homogeneous "matrix" in which a membrane is difficult to detect (fig. 25). It is necessary to consider whether the gray matrix is not due simply to tangential sections of closely packed viral particles, whether it corresponds to modified mitochondria as in erythroblastosis (*39*), or to a formation similar to the gray bodies found in myeloblastosis (*37*, *38*) which is a likely hypothesis. A relationship between these formations—the intravacuolar virus, the intracytoplasmic particles, and the sequence of a possible cycle of virus synthesis—is not yet clear.

As may be seen already, there is now no doubt that the application of tissue culture to the study of the Rous sarcoma, though in its very beginning, will provide a working system of most significant value for the attention of the electron microscopist. Already it may be said that:

1. Richness in viral particle number is sufficient to meet the requisites for easy study in the electron microscope.
2. No doubt exists that cytologically some evidence of developmental aspects of virus formation has been revealed.
3. The preparations obtained provide the possible means for developing a morphologic method for direct count of cell-associated virus particles which may immediately be related to biologic activity of similar samples.

CONCLUSION

Results of studies on cell ultrastructure permit only one conclusion: Without the presence of the viral particle itself, no cytologic lesion has been revealed by electron microscopy that will allow unequivocal inference of the viral origin of a tumor, at least with the present knowledge. The subject treated here was requested for this symposium for the precise purpose of presenting a comprehensive discussion of possible specific lesions to be found in virus tumors. It is of first importance to realize that the results are negative from the point of view of the host cell but, in contrast, are positive with respect to the agent insofar as experimentally induced tumors are concerned.

The implications of these observations, negative and positive, are already tremendous. It must not be forgotten that, as recently as 2 years ago, some virologists chose to adopt an austere attitude toward the findings by electron microscopy. The best that could be said, as quoted from one authority, was that ". . . certain kinds of particles are seen in one kind of tissue and appear to be absent in the other." A new phase of accomplishment has now been instituted in which the morphologic identification of viral particles with the electron microscope has become a valid method without which no further work on virus-induced tumors may be considered as complete (*83*). Impeding prejudices have been overcome. Previously, the morphologist, who was usually no virologist, had developed an inferiority complex because of the sad situation in which he had inadvertently found himself, either by discovering particles without being able to prove biologically that they were the causal virus or even virus at all, by not discovering them in highly malignant virus-induced tumor tissue, or still worse, by discovering them where he was not expecting them in control tissues. To each of these enigmas he now has an answer. He knows that some virus-induced tumors may harbor no particles at all and why, as in the Rous sarcoma. He knows, as illustrated for instance by the work on mouse mammary carcinoma, that the particle he observed in the tumors of the animal is the responsible agent. He also knows ways in which he can stimulate the development of particles, so that they will be numerous enough to be easily detected in the electron microscope. He even has a most plausible explanation for the presence of particles in "normal" controls. The virus particle, as it appears in tissue sections, has then regained its rightful place—after quite a battle. It has also acquired a unique value, since no other unequivocal sign of malignancy or viral involvement has been revealed.

In its turn, the absence of special cytologic features in virus-induced tumor cells, together with the frequent absence of particulate elements, is of fundamental importance in relation to the problem of human cancer. It has been shown and emphasized that thus far there is no ultrastructural difference between the cells of virus-induced tumors and the cells of a human cancer. Morphologically, on histologic as well as ultrastructural grounds, the research worker is in excellent position to accept the similarity. Experimentally, he is now in a situation where he can try to test the validity of the viral hypothesis of cancer with new techniques. No electron microscopy of cells from human cancer cultivated *in vitro* has been carried out extensively up to now, at least not to our knowledge. Attempts in this direction—the uncovering of corpuscular cellular elements—should be coupled with efforts to detect components perhaps present in a noncorpuscular or "molecular" state and comparable to genetic material (*83*). This other fundamental aspect of the problem cannot be developed in this discussion.

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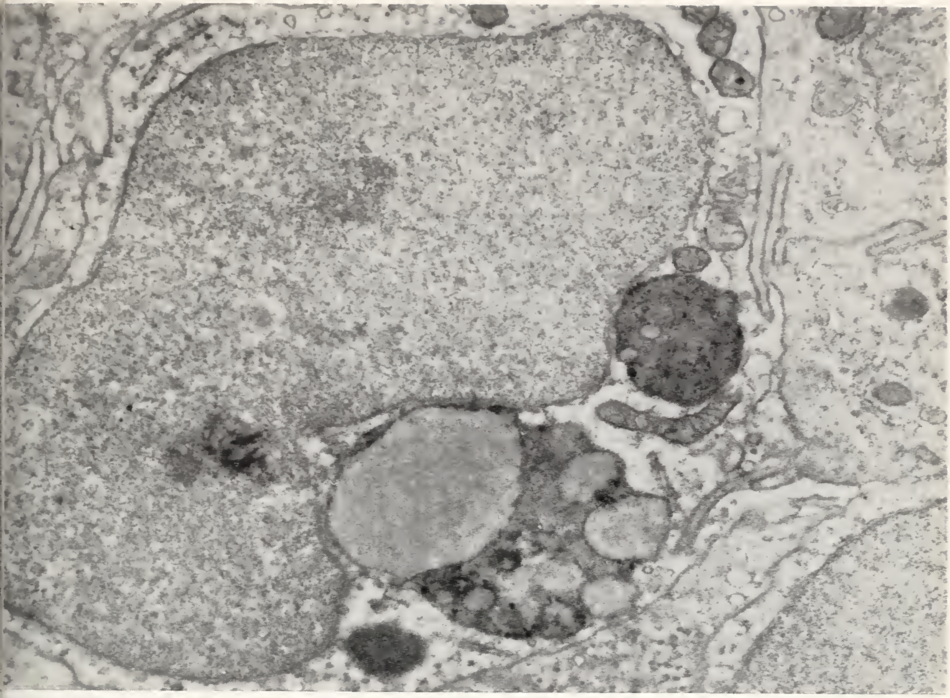
PLATES

Plate 25

Figure 1.—Normal-looking and relatively well-preserved nucleus of human breast cell. × 15,000

Figure 2.—Apparently badly preserved nucleus in cell from Rous sarcoma. *Note* margination of chromatin, patchy destruction of nucleoplasm, and presence of granules (→) of unknown significance. × 18,000



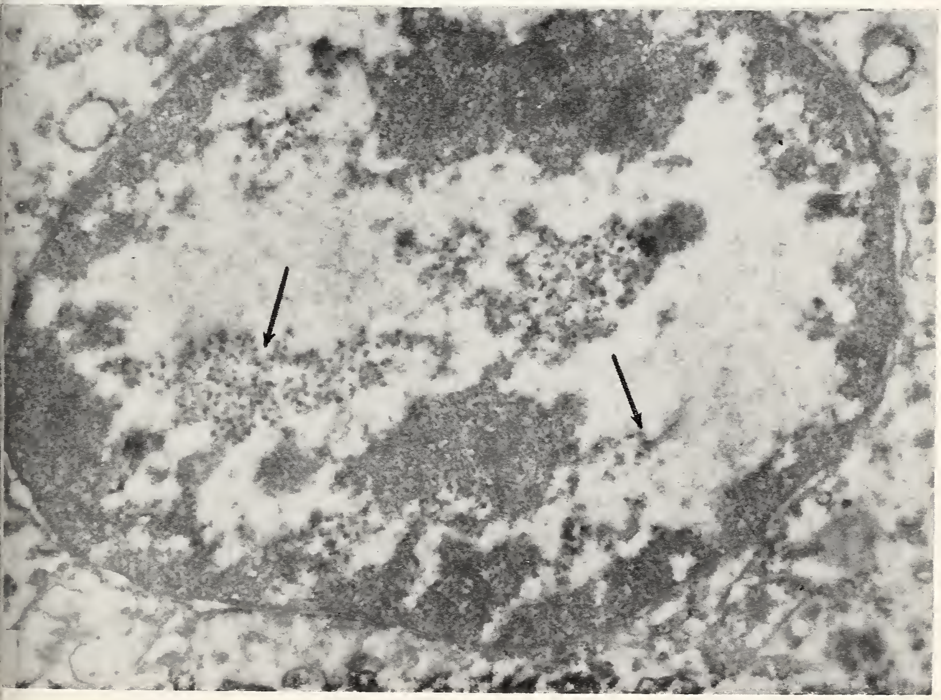
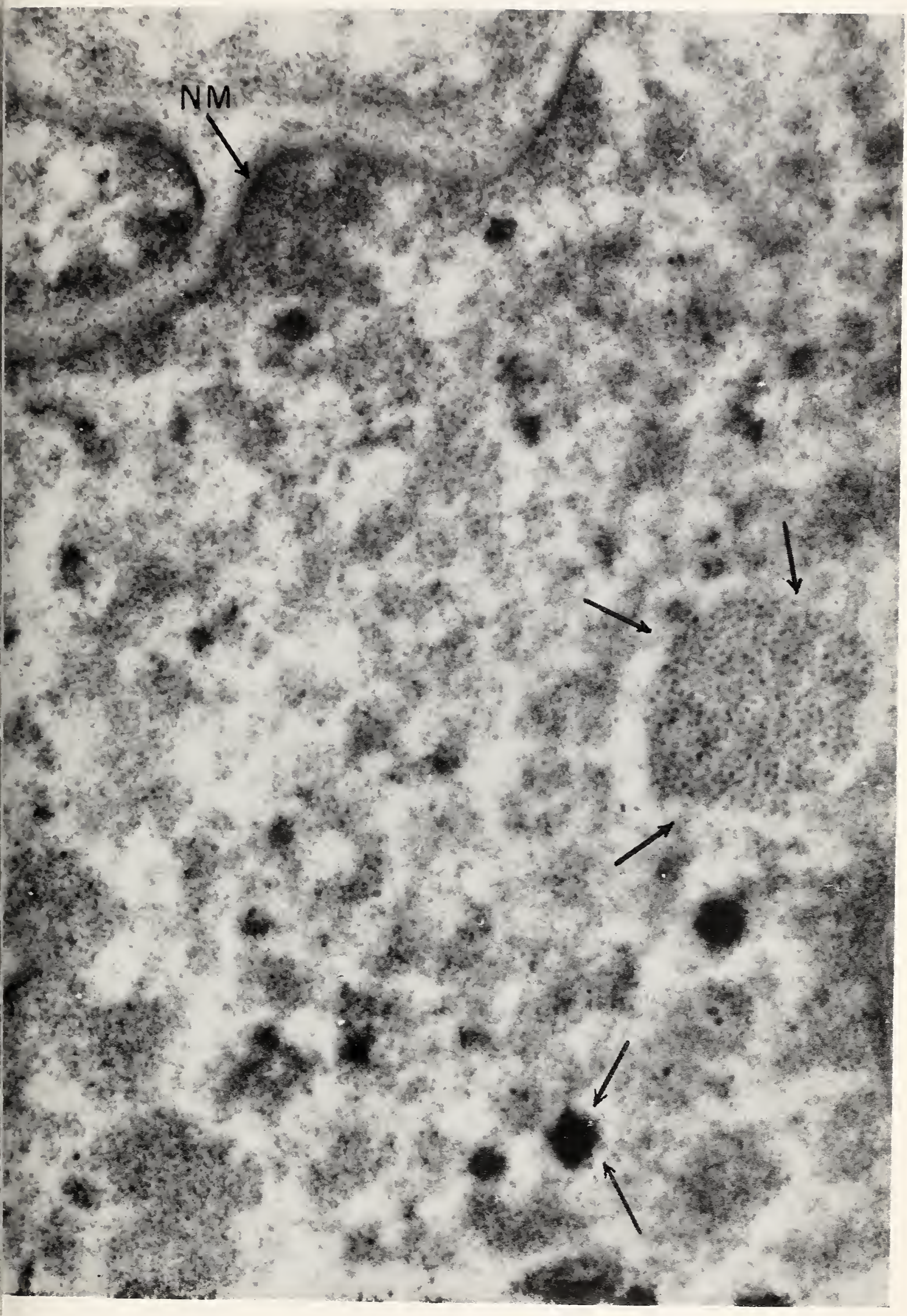


Plate 26

Figure 3.—Portion of nucleus of human breast carcinoma cell. *Note* aggregate of small granules and "dense bodies" (→) scattered at random in mottled nucleoplasm. Latter bodies are frequent in nuclei of virus-infected cells. NM—nuclear membrane. × 32,000



HAGUENAU

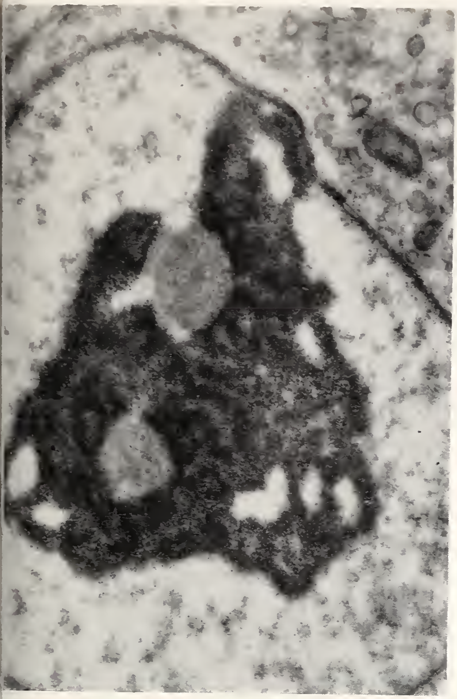
Plate 27

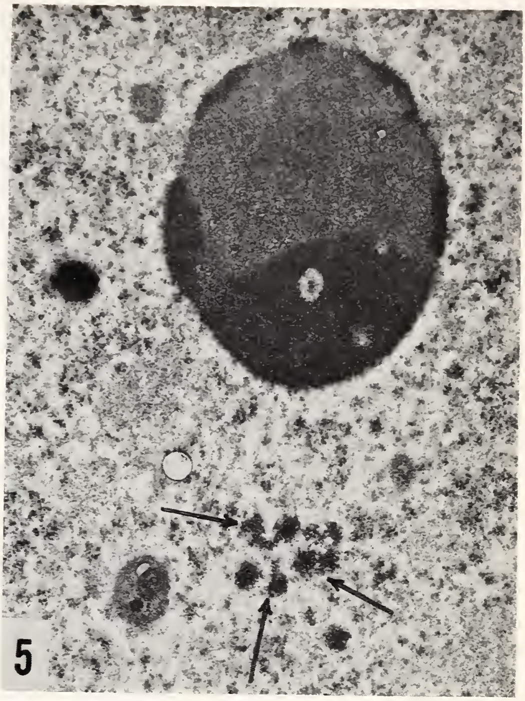
Different aspects of nucleoli in cancer cells

Figure 4.—Nucleolus of Rous virus-infected cell grown *in vitro. Note* "moiré" appearance of "nucleolonema" due to densification of some of its segments and accumulation of "pars amorpha" into small pools. × 15,000

Figure 5.—Pronounced condensation of nucleolar granules in human breast cancer—a frequent finding observed, also, in virus-induced tumor cells. *Note* "dense bodies" at (→). × 20,000

Figures 6 and 7.—Other examples of nucleoli in human breast carcinoma. With exception of figure 7, other aspects are not usual in normal cells, yet they probably correspond to modified metabolism and cannot be considered as specific. × 11,000





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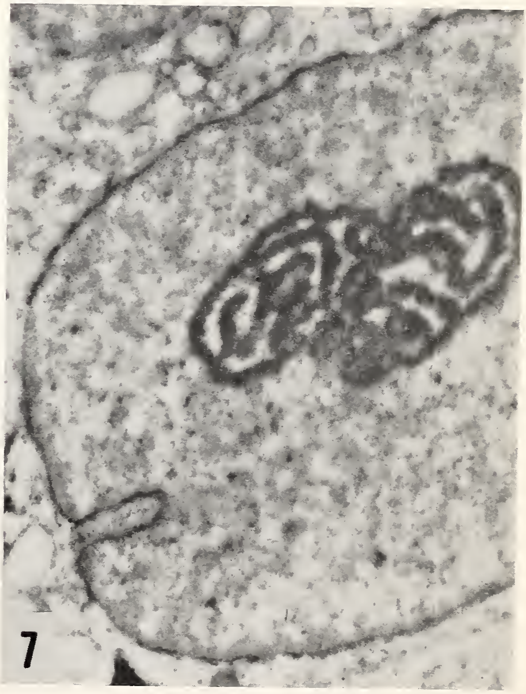


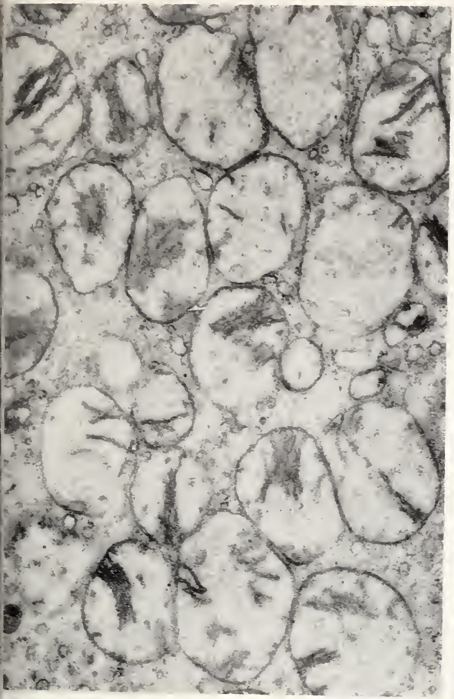
7

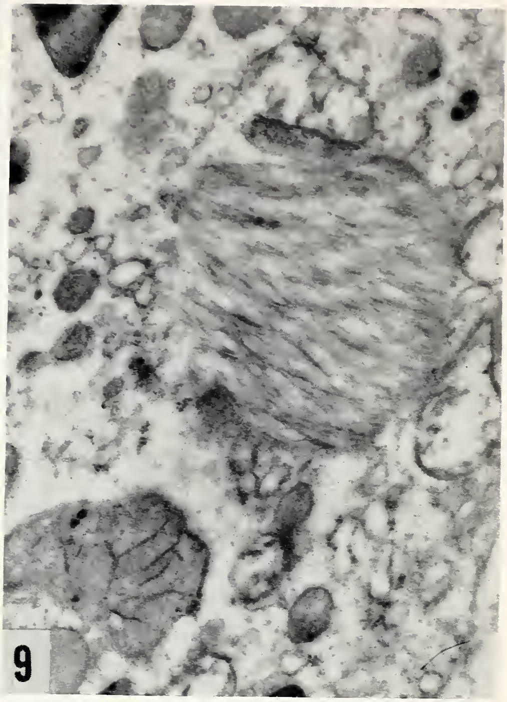
PLATE 28

Different modifications of mitochondria encountered in malignant cells. None of these changes is specific

FIGURE 8.—Illustration of cloudy swelling of mitochondria. × 30,000

FIGURES 9, 10, AND 11.—Other types of degenerative change in mitochondria. × 30,000





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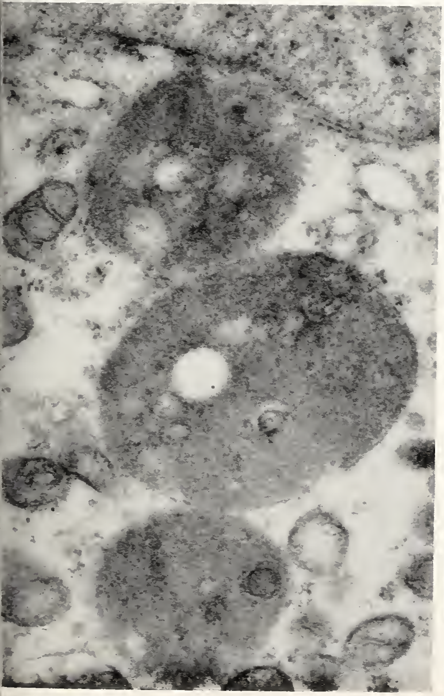

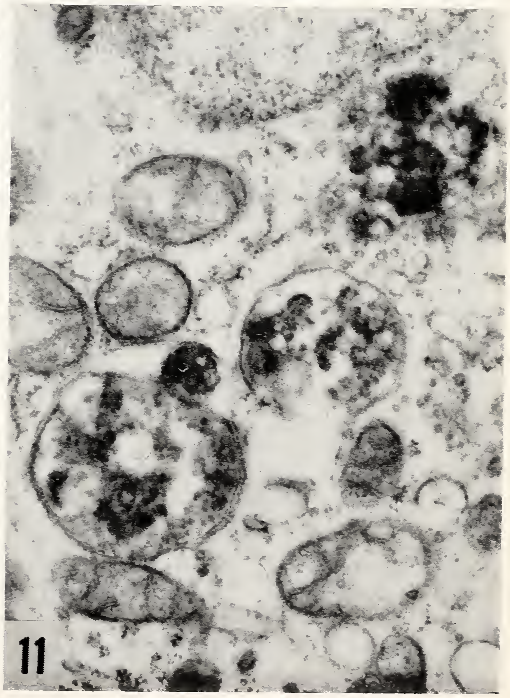

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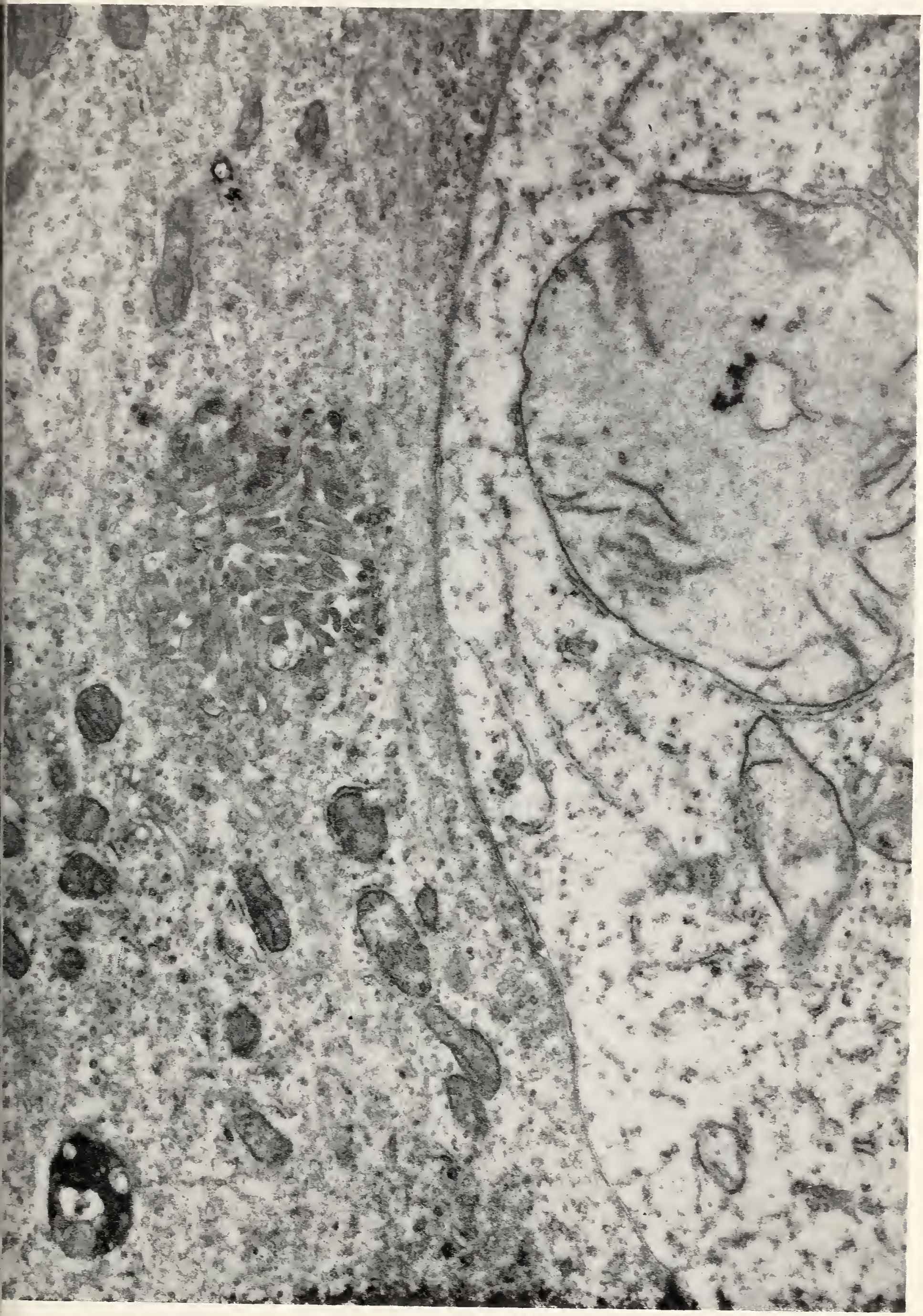
HAGUENAU

PLATE 29

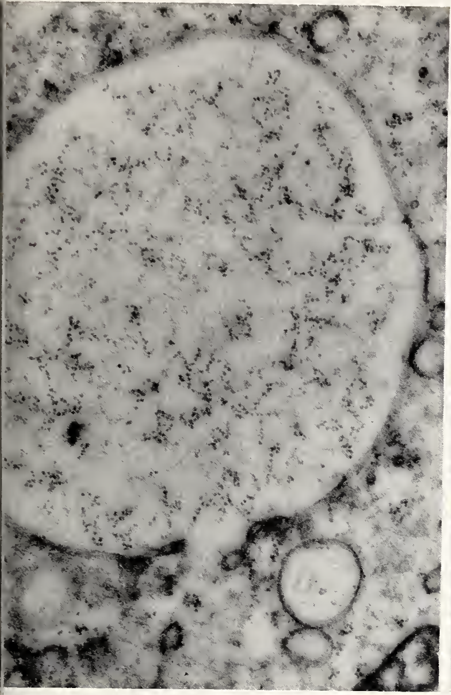
FIGURE 12.—Portions of 2 adjacent cells from human breast carcinoma. Mitochondrial swelling is illustrated at *right* and presence of intracytoplasmic canaliculae with hypervillosity is seen at *left*. These two features are frequently encountered in these malignant cells. × 18,000

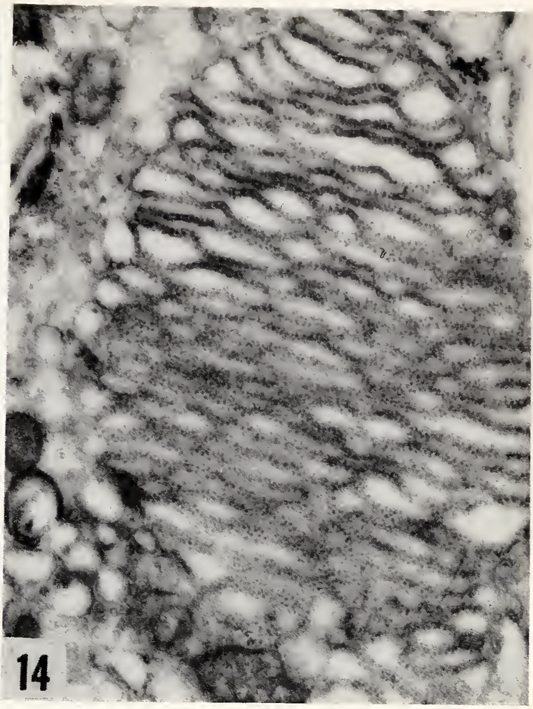


HAGUENAU

Plate 30

Figures 13, 14, and 15.—Different aspects of increase in ergastoplasm in Rous virus-infected cells. Accumulation of ribonucleoprotein granules in cytoplasmic vacuole (fig. 13), hyperplasia of lamellar (fig. 14), and granular (fig. 15) ergastoplasm in cytoplasm. × average 33,000





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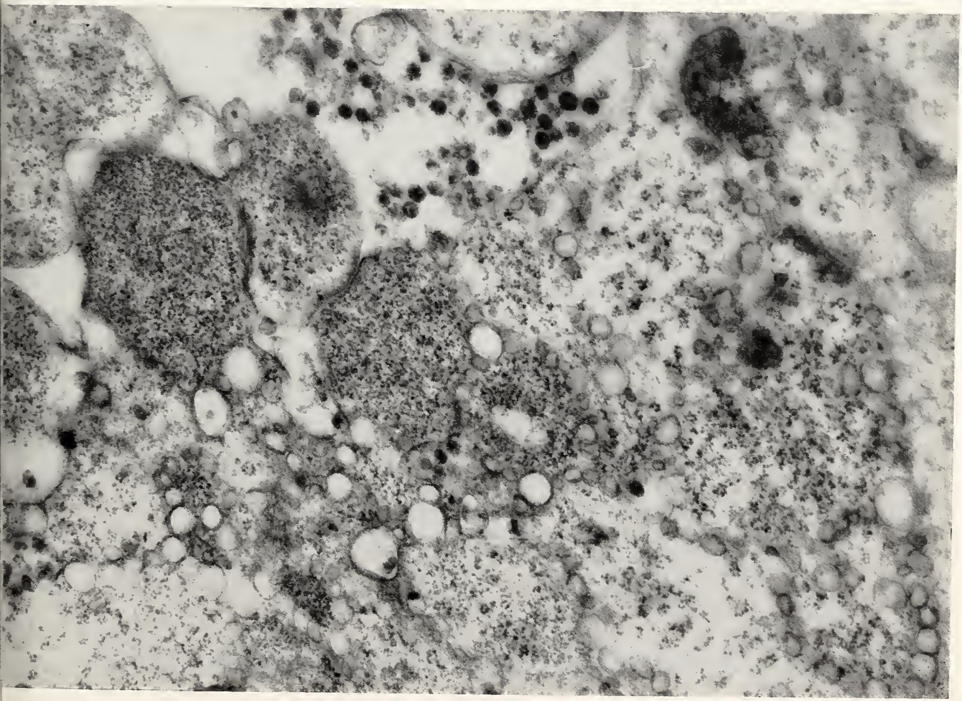
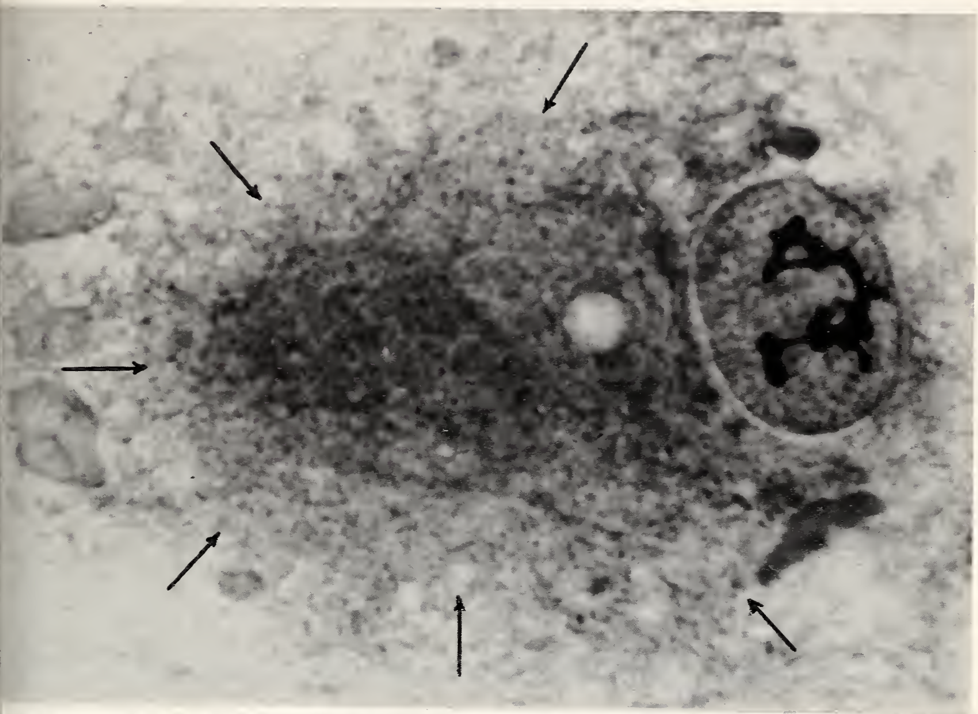


Plate 31

Figure 16.—Remarkably hypertrophied cellular center in Rous sarcoma virus-infected cell observed in optical microscope. × 2,000

Figure 17.—Ultrastructure of this region to show that it corresponds only to packing of Golgi vacuoles (→). No virus particles are associated with such hypertrophied Golgi apparatus. × 18,000



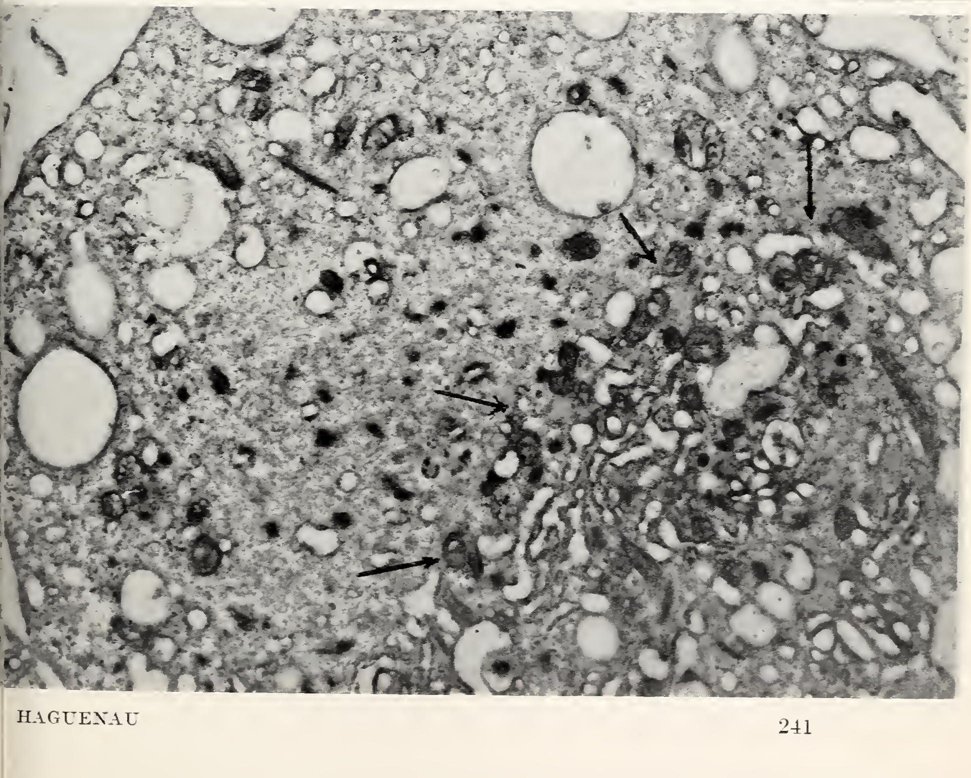


Plate 32

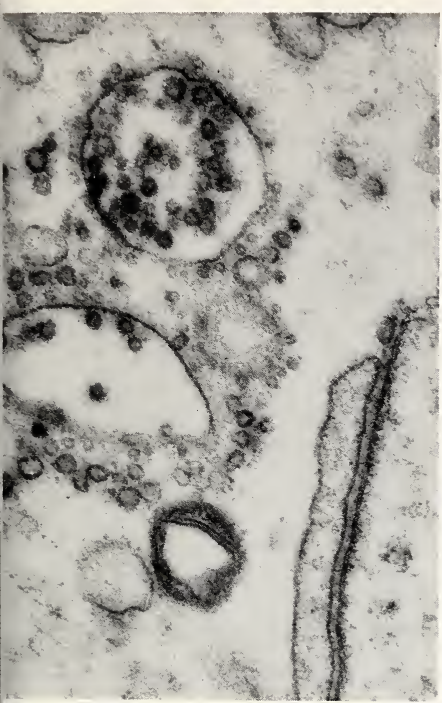
Diverse particulate formations to illustrate difficulties sometimes encountered in morphologic identification of viral particles. Only particles in figure 21 may be identified as such.

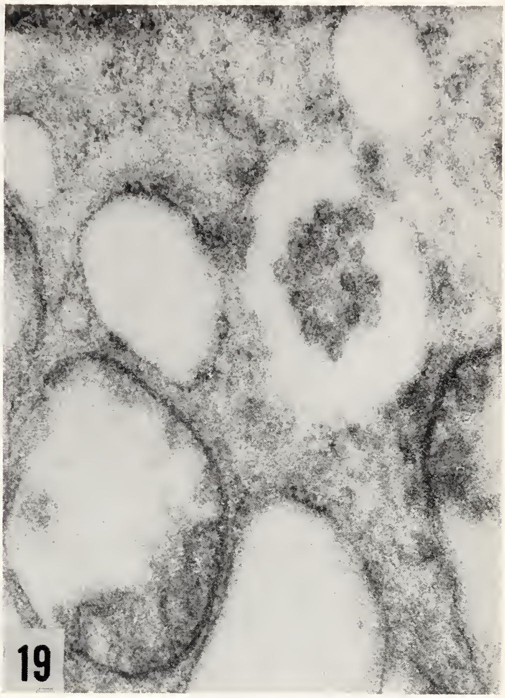
Figure 18.—Typical "compound vacuole" in testis of guinea pig (courtesy of J. André). × 42,500

Figure 19.—Particle-containing vacuole in human breast cancer. Significance of structure is unknown. × 30,000

Figure 20.—Nuclear "pores" from very indented nucleus in Rous sarcoma cell. × 40,000

Figure 21.—Purified preparation of Shope papilloma virus particles. × 120,000





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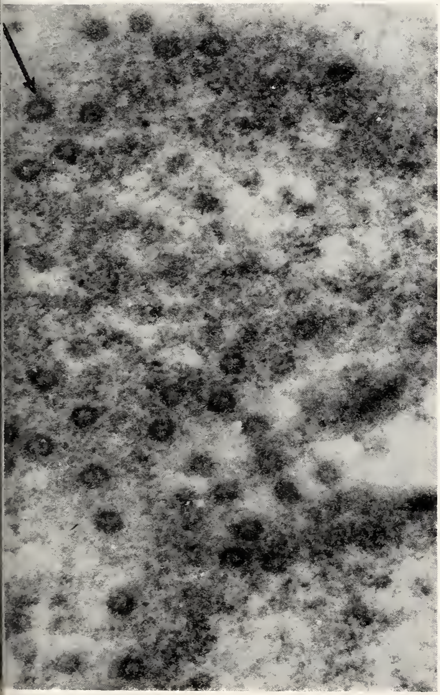

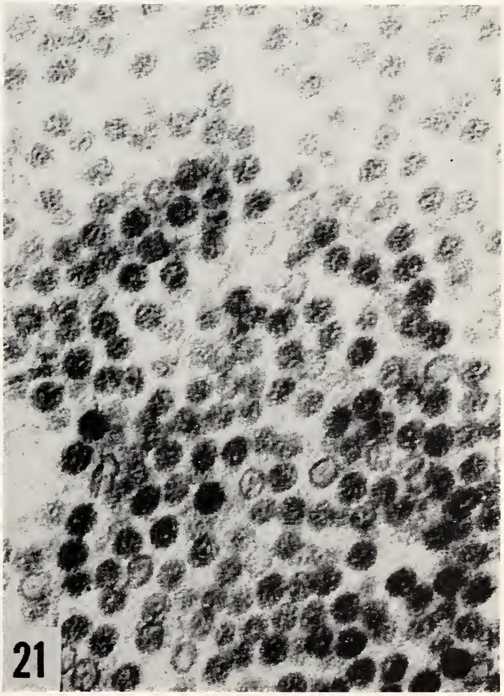

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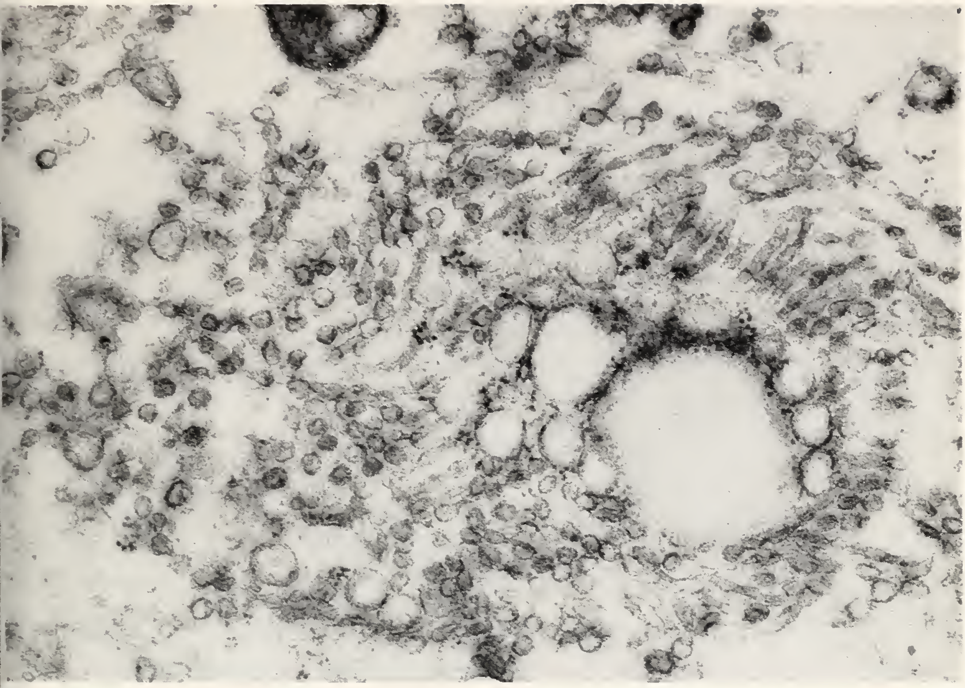
Plate 33

Figure 22.—Increase in cytoplasmic vesicles (endoplasmic reticulum) in Rous virus-infected cell. × 51,000

Figure 23.—Pinocytosis vesicles and their relationship to surface membrane and to intracellular vacuole in Rous virus-infected cell. Virus particles have probably been engulfed through this process. × 40,000



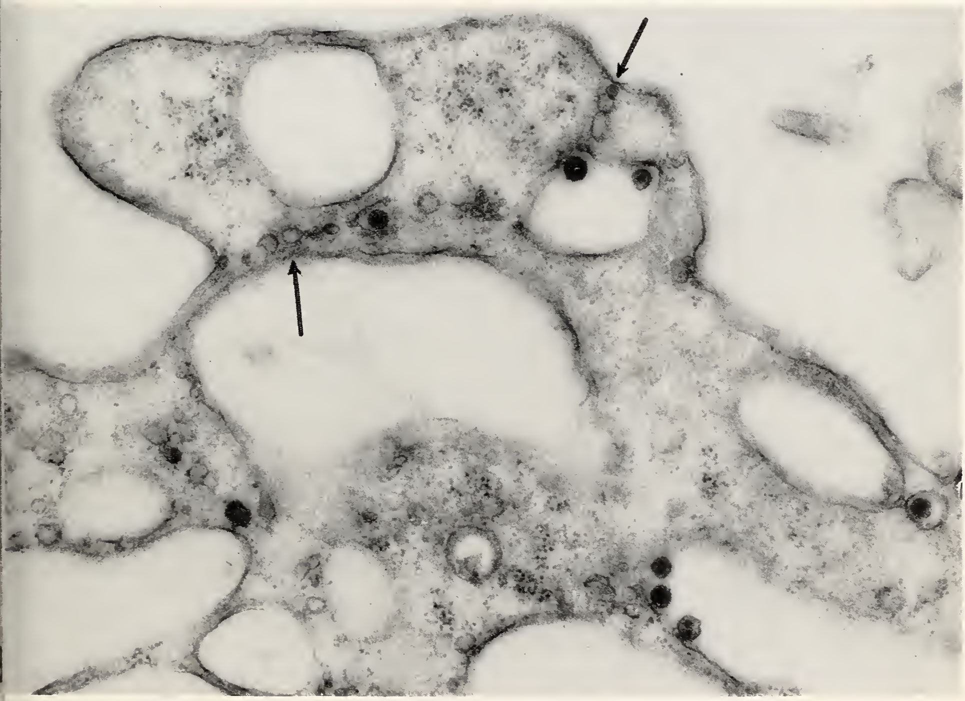
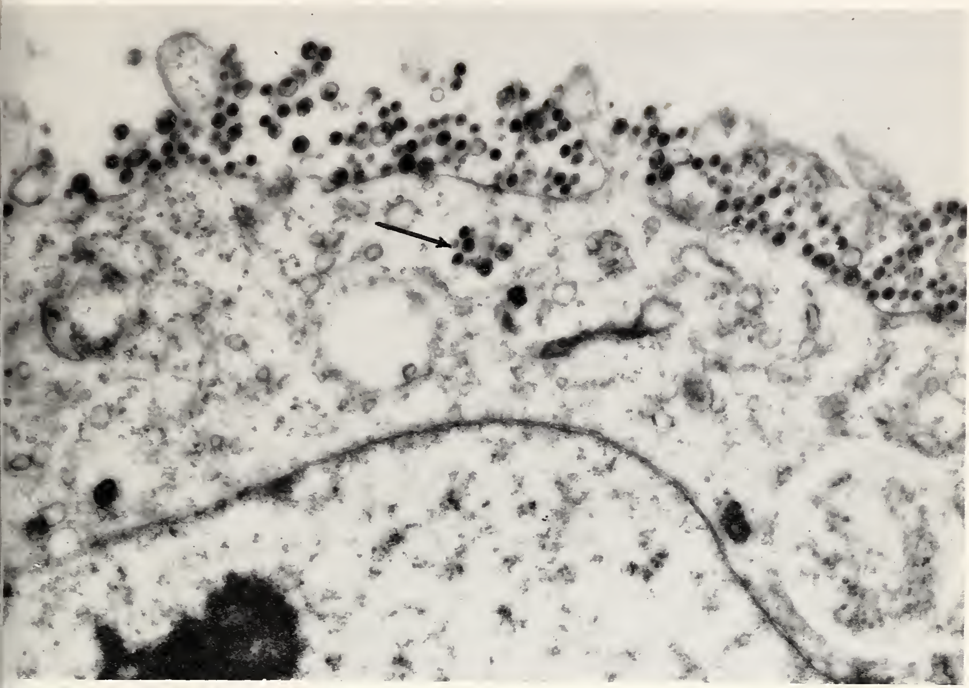
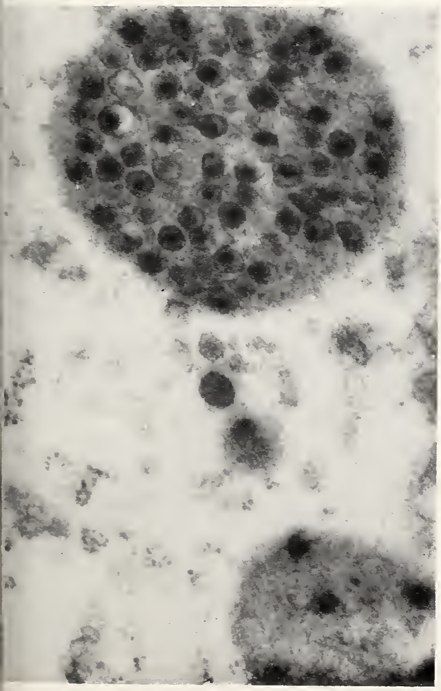
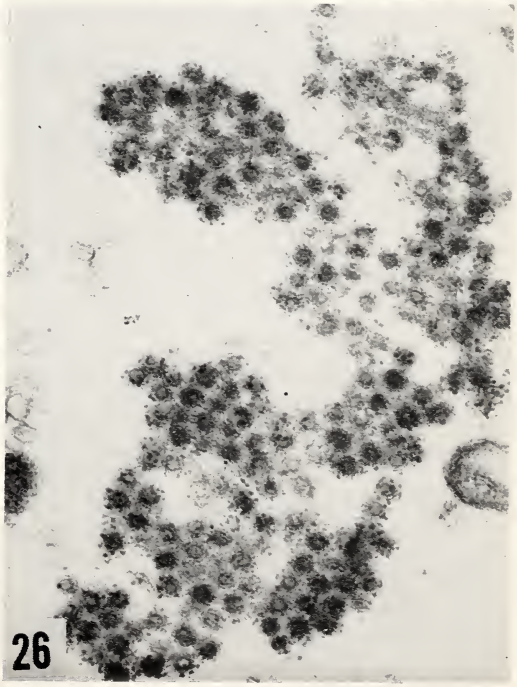


Plate 34

Figures 24, 25, and 26. Extracellular (fig. 24) and intracellular (figs. 25 and 26) types of virus particles found in cells infected with Rous sarcoma virus *in vitro*. Classical appearance is illustrated *above*. Virus is present at cell membrane surface or inside clear vacuoles (→). In figure 25, the "gray body" type of formation differs from usual empty vacuole, while in figure 26 a totally different type of particle is demonstrated. Its relationship to the other is still unknown. × 42,000







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Discussion

Dr. Dalton (National Institutes of Health): Dr. Haguenau, what is your interpretation of the multivesicular bodies which you described? Do you consider them to be a fairly regularly occurring component in relation to virus infection?

Dr. Haguenau: It would appear that they are probably part of the Golgi apparatus in normal cells, but the ones seen in human breast cancer do not quite fit this description.

Dr. Dalton: Do you regard the presence of Rous virus particles in vacuoles as always the product of phagocytosis?

Dr. Haguenau: Yes, I think that those in true vacuoles have been engulfed by the cell rather than formed there in this particular material *in vitro.*

Dr. Gaylord (Yale University): I was amazed to see Dr. Haguenau's pictures of the structures in the Rous sarcoma cell nuclei. It is difficult to understand why they have not been seen before, since they are so conspicuous. Were those tumor cells or cultured cells?

Dr. Haguenau: The larger granules which I showed were in the cytoplasm and not in the nucleus. In the nucleus there were only small granules, perhaps in the range of size of polyoma virus. These are present in Rous tumor cells.

Dr. Gaylord: Were the patches of small particles numerous?

Dr. Haguenau: They were in cultured cells. The "dense bodies" have not been *in vivo.*

Dr. Fogh (New York State Health Department): It is sometimes difficult for a nonprofessional morphologist to understand your criteria for designating a particle as virus. Is it ever possible from a purely morphologic observation to identify a particle as virus?

Dr. Haguenau: Yes. Some of the viruses have very definite features which distinguish them from any other type of particles. For instance, the avian viruses are all alike, and there is no doubt that they have a nucleoid and a double membrane and that there is no other structure in the cytoplasm of the normal cell which has the same aspect.

Dr. Howatson (Ontario Cancer Institute): I would like to ask Dr. Haguenau whether she has found in the polyoma virus any evidence of formation of the particles in the cytoplasm, particularly within mitochondria or bodies derived from mitochondria.

Dr. Haguenau: I have not really worked with this virus, but I know that Bernhard has seen particles in the cytoplasm. I have never seen a picture of his in which there appeared to be an actual site of formation in the cytoplasm. Have you?

Dr. Howatson: We think we have.

Dr. Parsons (Oak Ridge National Laboratory): I would like to confirm Dr. Haguenau's observations about the compound vacuoles, particularly in cancer tissue. In a study of 13 cases of acute leukemia and Hodgkin's disease, this was one of the most striking features of these tissues as compared with normal white cells. These particular structures, little vesicles inside large ones, which Law has called the compound vacuole, were very prominent, particularly in Hodgkin's disease. It would appear that the term "multivesicular bodies" should be limited to primitive leukocytes. This was a term given by Porter to a very distinct and specific structure in leukocytes which, as far as I know, is not seen anywhere else.

Dr. Fogh: In an electron microscopic study made in collaboration with Dr. Edwards, in which we studied sections of normal amnion cells in the membrane, of primary amnion cells in primary culture, and of transformed amnion cells, we saw the multivesicular bodies only in the transformed cells.

Dr. Dalton: In your material in tissue culture, have you found an apparently definite life cycle of the Rous virus, as the group at Duke have shown in relation to the gray bodies in the development of myeloblastosis? In some material from one of Dr. Bryan's chickens that Dr. Ziegler has been looking at, we found particles in the lumen of the pancreas in young chicks. The sections showed a budding of these particles from the surface of the cell membrane just as one finds in other tumor cell types, for example, as with the Friend agent.

Do you feel that, in the tissue-culture studies on Rous virus, you are dealing with a special situation and a special environment, or do you think that the Rous agent is different from this unknown avian virus which we saw?

Dr. Haguenau: Do you really think that this is a typical Rous virus particle that you are referring to?

Dr. Dalton: I am only claiming it to be an avian virus, because it is in a chick.

Dr. Haguenau: I do not think that your pictures resemble the typical Rous virus particle. Your particle has no dense nucleoid or double membrane around it. That it seems to bud off is very unusual for the Rous virus, but may be related to the fact that you are dealing with an epithelial cell instead of a mesenchymal cell; that I do not know, but this story is not clear.

Dr. Beard: Dr. Dalton's picture is much like that seen in erythroblastosis. I would guess offhand that his particles were not Rous virus at all but erythroblastosis.

Dr. Banfield (National Institutes of Health): Dr. Haguenau, would you say more about the particles that you saw in your examinations of human breast cancer—where they occur, and their size? How often have you seen them and have you ever found dark bodies in the nuclei or anything that you might think were these dark bodies?

Dr. Haguenau: Yes, but this is a very infrequent finding. I have seen it about 3 times out of about 100 cases which we have examined. However, it was only by looking at photographs afterwards that I discovered them. I never looked for them on the microscope screen. It may well be that they are more frequent. Nevertheless, when they are seen, they are always in the nucleus and associated with the dense bodies. There are both the granular kind of structure and the dense bodies in the nucleolus, too, in human breast cancer.

Dr. Parsons: I would like to ask Dr. Haguenau her impression of the present status of osmic acid fixation, that is, whether she thinks the osmic acid gives a good appearance of the interphase mammalian nucleus or whether there is more that can be learned by some other staining technique. It seems that osmic acid is deficient in some way in staining the nucleus, particularly because of recent reports dealing with leukemic cells as having very abnormal chromosome configurations. If fixation could be improved, it might be possible to distinguish something definite between cancer cells and normal cells.

Dr. Haguenau: This is evident, and we would very much benefit by the use of other fixations for the study of nucleus, but at the same time, I do not feel that osmic acid fixation gives a distorted image. With our actual notion of the structure of the interphase nucleus, the idea of very thin coiled filaments cut tangentially in many sections does fit with the image we get.

Dr. Gey (Johns Hopkins Hospital): I would like to ask whether, in differentiating normal and tumor cells, you believe, as I do, that in the malignant cells, regardless of whether or not they are infected with viruses, one would find altered and decreased number of mitochondria and whether, in many malignant cells, there is not also a feedback mechanism involved in the uptake of materials from degenerating cells into competent cells which do take up these materials.

You mentioned something about some myelin figures and other structures. Do you not also identify many particles that have come from other cells?

Dr. Haguenau: Yes. Concerning mitochondria there is a definite answer. We have recently studied rather extensively the renal carcinoma of man, the clear-cell type, in which one of the most striking features is the amazing increase in mitochondria. Although the kidney tubule cell is full of mitochondria normally, the most striking feature in this growth is that nearly the whole cytoplasm is filled with mitochondria. Thus, instead of a decrease in number, usually considered particular to cancer, there are remarkably large numbers of mitochondria in this growth.

With respect to the turnover and engulfment of substance that might come from other cells, I am sure that this may occur, but I do not know of pictures that illustrate it.

Virus of Avian Myeloblastosis. XVII. Morphology of Progressive Virus-Myeloblast Interactions *In Vitro* [1,2]

R. A. BONAR, DAVID WEINSTEIN, J. R. SOMMER, D. BEARD, *and* J. W. BEARD, *Departments of Surgery and Pathology, Duke University Medical Center, Durham, North Carolina*

A major development in the recent study of virus tumors has been the demonstration of virus particles in association with tumor cells and the physical identification of ultrastructural cell-virus interrelationships. The findings, obtained primarily by the electron microscopic examination of thin sections, have revealed the most varied aspects of cell-virus interaction concerned with virus synthesis. These are now known (*1, 2*) to extend through a spectrum of cell involvement from definitive changes associated with the nucleolus (*3*), with the total nucleus (*4*), with nucleus and cytoplasm (*5*), with cytoplasm alone (*6–9*), and, finally, to the participation of the cell membrane (*10, 11*) in the formation or shedding of virus from the cell. Such advances have been an achievement not only in the understanding of morphologic relationships but have provided much physical clarification of previously completely obscure biologic phenomena.

Of greater import than the present status of the problem, however, is the promise of fruitful outcome of further investigation of cell-virus relationships at levels increasingly near those of the biochemical reactions concerned with synthesis of virus and influence of the agents on their respective host cells. The problem of attaining these objectives is presently subject to numerous impediments, both technical and biologic. Resolution with the electron microscope, though great, is decidedly limited with the present techniques of fixation and staining. The obstacles are by no means insurmountable, since much effort is being expended with success (*12*) to extend the range of size and constituents possible for visualization. In addition, as yet but little exploited, is the whole system of cytochemistry, which is certainly applicable at the level of light microscopy (*13, 14*) and much of which may be adaptable to electron microscopy (*12, 15–17*).

The biologic limitations constitute a problem of a different order. Analysis by electron microscopy is dependent on resolvable ultrastructure

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[1] Presented at a Symposium on Phenomena of the Tumor Viruses, New York, N.Y., March 25 and 26, 1960.

[2] This work was aided by research grants to Duke University from the American Cancer Society, Inc.; from the National Cancer Institute, National Institutes of Health, Public Health Service; and by the Dorothy Beard Research Fund.

(*18*) specific to the infected cell. In the same way the outcome of cytochemical study is related to the quantitative aspects of distribution and concentration of specific reactant materials in the cell. Some control or alteration of these biologic relationships has been attained, particularly by tissue culture of tumor cells (*10*) and by special host-virus adaptations (*19*) which have greatly enhanced opportunities for the study of such difficult systems as the Rous sarcoma virus (*20*). The steady progress in this area gives cause for much optimism that, as in the sphere of physical and biochemical techniques, biologic difficulties may be overcome.

A virus-tumor system providing particularly favorable conditions for investigation of virus-host cell relationships is that of the virus-induced avian leukemia, myeloblastosis (*21, 22*). The myeloblasts of primitive aspects and neoplastic behavior appear in great numbers in the circulating blood of chickens with the disease. Under proper conditions, these myeloblasts from the chicken proliferate at exponential rates for indefinite periods in tissue culture (*23*). Moreover, treatment of normal bone marrow *in vitro* results (*24*) in growth in culture of cells indistinguishable from myeloblasts obtained from the diseased chicken. Concurrent with growth, the cells liberate the etiologic virus into the culture fluid at rates related to cell characteristics and the conditions of culture. Since the cells flourish in fluid suspension, growth can be quantitated by simple cell count. A special advantage for quantitative studies on virus synthesis and liberation of virus is the measurement of absolute numbers of virus particles by estimate of the highly stable adenosinetriphosphatase activity of the agent (*25–27*).

Of particular importance for the present report has been the observation, by electron microscopy (*9*), of definitive cell ultrastructures specific and, under some conditions, quantitatively related to infection of the cell and liberation of the virus. It has been observed, also, that these ultrastructures are of a size applicable to study by light microscopy and to well-developed systems of cytochemistry (*14*). As a result the opportunity has been afforded to apply the techniques of electron and light microscopy for correlated studies, together with observations with tissue culture, aimed at the clarification of the dynamic aspects of cell response to the agent. Many of the findings encountered in the work have already been described (*9, 14, 24*). More recent investigations have confirmed and clarified previously somewhat obscure elements in the sequence on relationships in the origin of the specific structures and their bearing on virus formation. These results, obtained by electron and light microscopy, will be discussed in their bearing on the biologic phenomena of tissue culture reported in an associated publication (*23*).

MATERIALS AND METHODS

Myeloblasts were obtained for immediate study and for tissue culture from the circulating blood of chickens with myeloblastosis. The virus employed to produce disease was the BAI strain A (*28*) inoculated intravenously into line 15 White Leghorn chickens (*29*) at 5 to 10 days of age. The methods used for collecting the myeloblasts from the blood and from tissue culture, as well as the techniques of establishing and maintaining the cells in culture, have been described in another report (*23*).

Cells for electron microscopy were fixed in 1 percent osmium tetroxide in Veronal acetate buffer, *p*H 7.4, for 1 hour at 2° C., dehydrated in graded concentrations of ethanol, and embedded in a mixture of methyl and butyl methacrylates in the proportion of 1:4. Sections were cut with a glass knife on a Porter-Blum microtome. Some sections were floated on lead subacetate to enhance the contrast (*15, 16*). Electron micrographs were taken with RCA microscopes EMU 2D and EMU 3D.

Cytochemical analyses were made on cells from the bone marrow of normal chickens, from the circulating blood of chickens with myeloblastosis, and on cells from tissue culture. This report is concerned with the results of studies on living cells by phase-contrast microscopy and with examination of the same cells after they were stained for adenosinetriphosphatase activity with the Wachstein-Meisel reagent (*30*). A small drop containing myeloblasts obtained by centrifugation from blood or directly from tissue-culture fluid was placed on a coverslip, which was dropped onto a slide with a corner projecting over the edge. The size of the drop was such that the spread did not quite cover the entire area of the coverslip, and, consequently, there was enough pressure to flatten the cells. After examination of indexed groups of cells by phase contrast (*14*), the preparation was plunged into liquid nitrogen for 30 seconds, the slide was removed from the fluid, the coverslip was pried off, and the slide was placed in a tube immersed in liquid nitrogen which was immediately evacuated. Afterward, the tube was transferred to an alcohol-dry-ice mixture, and the preparation was dried at this temperature *in vacuo* for 1 hour. Evacuation was continued while the tube was taken from the freezing bath and warmed to room temperature. From this stage, the dehydrated cells were fixed at room temperature in formaldehyde vapor for 3 minutes before application of staining procedures, or were stained without other fixation treatment. Staining for adenosinetriphosphatase was carried out essentially as described by Wachstein and Meisel (*30*). The substrate was adenosine-5′-triphosphate, and the period of incubation of the preparation was 2 hours.

RESULTS

The work described here was of 3 principal aspects: (1) continued study of myeloblasts of the circulating blood; (2) determination of the progressive ultrastructural changes in virus-associated myeloblasts under different conditions; and (3) refinement of technique and extension of cytochemical studies on myeloblasts and the cells of normal bone marrow. It has been the principal aim to follow the ultrastructural changes in myeloblasts in correlation with the time and varied conditions of culture.

Ultrastructure of Circulating Myeloblasts

The infected myeloblasts from the circulating blood of chickens with this form of leukemia resemble closely, figure 1, the blast cells found in the bone marrow of normal birds of the same age. There is seen a large nucleus with a prominent nucleolus and often a nuclear notch which is best demonstrated by phase contrast as described in another section. A Golgi zone is located near the notch and consists of small vesicles and a few flattened sacs. A section usually includes several mitochondria and, also, several smaller round or ovoid bodies, designated earlier (*9, 31*) as gray bodies which probably are analogous to the microbodies, globoid structures, thought (*32*) to represent mitochondrial precursors. The endoplasmic reticulum is rather sparse and appears chiefly as small vesicles with a few longer profiles. Many ergastoplasmic granules are free in the cytoplasm. Recognizable virus particles occur in these sections only rarely, usually singly in small vesicles or vacuoles.

It is well to call attention in figure 1 to the presence of several vacuoles. As can be seen in phase contrast or after supravital staining with neutral red, such structures are arranged in a rosette pattern in or about the Golgi region. For the most part they are rather large and, in contrast to the appearance of monocytes under the same conditions, are relatively uniform in size. No difference is evident between the vacuoles of the normal myeloblast and those of cells from tissue cultures or circulating blood in size, staining reaction with neutral red, number, or distribution. Not all vacuoles are in this region, since a few may be scattered in other parts of the cells. In the vacuoles of myeloblasts associated with the virus, there may occur, as evident in figure 1, numbers of amorphous, darkly staining particles and, rarely, in the cells from the circulating blood, one or more virus particles of typical appearance (*2, 33*). It is notable, also, that there exists no definitive criterion for distinguishing every micro- or gray body from other structures of various sizes and similar staining reaction to osmium tetroxide.

Myeloblasts From Cultures Inadequate to Support Growth

As described earlier (*34*), myeloblasts may be maintained for long periods in culture media consisting of 20 percent chicken serum in a balanced saline solution in which the rate of cell multiplication is approximately the same as the rate of cell death. Under these conditions, despite the lack of increase in the cell population, there occurs a continuous release of myeloblastosis virus into the culture medium at rates varying from about 5 to 40 virus particles per cell per hour (*23*). Examination of thin sections of such cells reveals no feature, with the possible exception of larger numbers of small gray bodies, distinguishing them from cells studied immediately after removal from the circulating blood. Another possible difference is a tendency toward increased distinctness of cellular structures of cells from culture as compared with those taken directly from the bird. This impression would be difficult to quantitate, but the interpretation seems justified on the basis of examination of many sections of these cells. The state of the cell taken directly from the chicken may possibly be related to a relatively poor nutrient state of the blood of the leukemic bird (*35*).

Myeloblasts From Growth-Supporting Culture Medium

In the foregoing description much emphasis has been given to the lack of evidence of virus or material identifiable with virus-derived or associated structures in the myeloblasts in the blood or in culture under certain conditions. Since such cells are not distinguishable in this respect from myeloblasts in the bone marrow of the normal bird, they may be regarded as providing a "control" basis of ultrastructural characteristics suitable for qualitative and quantitative distinction of progressive or regressive change in the dynamics of cell response to the agent in relation to differences in environment. The capacity of the cells to undergo an enormous development of ultrastructural specificity has already been described (*9*), but there was noted a large variation in the findings not understood at the time. It is now recognized that the differences from one study to another were associated with variation in tissue-culture medium which has recently become subject to control (*23*, *36*). Under these conditions, the characteristics of ultrastructure are much more uniform and predictable.

Myeloblasts from the blood established in a medium of 50 percent chicken serum in medium 199 supplemented with B vitamins, including a high concentration of folic acid (*36*), exhibit change clearly evident within 2 hours. This is marked, first, by the increase in number, size, and appearance of the structures designated as gray bodies or viroplasts (*9*). The initial stages in the progression of change are well illustrated in figure 2, which is a section of a myeloblast of the same stock as that of figure 1 after 2 hours in tissue culture. There are now present 3 large gray bodies or viroplasts (PL) of a sort not seen in cells fresh from the circulating blood. In addition, other smaller bodies of somewhat similar appearance are scattered through the section, particularly at the upper right. Such bodies, both large and small, were numerous at this 2-hour interval of culture, but rare after only 1 hour. Nothing else was outstanding, except evidence that some mitochondria presented the appearance of degenerative change (AM). Other and by far the majority of mitochondria were essentially unchanged except for some possible swelling. The structure of the nucleus, nucleolus, Golgi region, and other parts of the cytoplasm showed no identifiable alteration.

During the initial 24-hour culture period, the progression, figure 3, comprises, in part, an increase in the number of viroplasts in the individual cells, practically all of which can be seen thus to be involved. In addition, many of the viroplasts show evidence of virus particles of typical characteristics, as well as bounded circular bodies of the size of the virus, but without the densely stained central region. It should be emphasized that the viroplast structures may differ greatly in size and appearance, a variation of much importance for later interpretations of cytochemical findings. In figure 3 there is a large viroplast of relatively homogeneous staining in which there is a single peripherally located virus particle. Another structure characteristic of the very early cultures is the "blossom" form (*9*), evident in great profusion in the cell from a 48-hour culture shown in figure 4. These, as seen frequently, contain both typical virus particles and those without dense centers.

It is notable in figure 3 that in the cell of the 2-hour culture there is evidence of extensive mitochondrial alteration (AM), though some of these organelles contain well-defined cristae and membranes. While this cannot be fully ascribed to specific involvement by the virus, there scarcely seems to be any other precursor for the large number of viroplasts present in early culture cells like the myeloblast of figure 4. In this cell there is a broad spectrum of forms of viroplasts (PL) in which well-defined virus particles (VP) are to be seen. It is remarkable to note the numerous mitochondria of entirely normal appearance despite the profound involvement of the total cell and the occurrence of many altered mitochondria.

As the cells are continued in culture in a medium adequate for growth, there is further progression of structural changes associated, in the rapidly growing cell population, with intensification of cell response to the virus. The process of ultrastructural stabilization extends through approximately the 4th to the 6th day, to yield cells with the appearance of the cell seen in figure 5 which is a myeloblast after 8 days in the growth medium, *i.e.*, a cell from a culture established 8 days before. After about the 6th day the densely gray viroplasts seen earlier are only rarely present, but instead, there is a variety of forms seemingly representative of a spectrum of structures that range in organizational complexity from mitochondria (AM), which appear to have lost some of their internal regularity, through bodies designated as viroplasts (PL), on to empty vacuoles. In one structure (AM) near the nucleus at the left, there are seen definite cristae at one end and a viroplast-like appearance at the other. This sequence of structural variation is observed for indefinite periods under the conditions described.

The vacuoles vary considerably in size and in the amount of internal stainable material. Some that contain one or more virus particles may be seen near the edge of the cell and may give the impression of being just about to open or of having just opened to the outside. Figure 6 shows an unusually large structure apparently of this nature which contains many virus particles. The inclusion is surrounded by a membrane that seems distinct from the cell membrane but close to it (100–200 Å) over a considerable distance. Such a structure containing so many virus particles is but rarely found. Another large group of virus particles lies in the space between the three myeloblasts shown.

An outstanding feature of myeloblasts in culture is their capacity to multiply for indefinite periods under proper conditions (*23*). In a medium of 50 percent chicken serum in medium 199 supplemented with vitamins, containing particularly high concentrations of folic acid, the generation rate varies from about 3 to 8 days. It seems remarkable that these cells obviously extensively involved in response to the etiologic agent in the processes of virus synthesis should display such a capacity to engage, at the same time, in the mechanisms resulting in rapid cell multiplication at exponential growth rates. The magnitude of cell involvement is shown by the presence of relatively large numbers of viroplasts and related bodies and, more evident from the dynamic aspects of the relationship, by the prolonged output of virus particles at rates of about 40 particles per cell per hour. Furthermore, and fully as impressive, is the scant evidence in thin sections of damage to heavily involved cells and by the small number of cells taking trypan blue stain in the routine counting of culture cells. In the latter case the number of such elements was less than 5 percent of the total population.

Mitotic figures of normal features were numerous in fixed and stained preparations of culture cells and were seen occasionally in thin sections, as illustrated in figure 7. Here there are evident the centriole pair (C) from which there extend the spindle fibers (SF) toward the disseminated chromatin material (CH). It is seen that numerous viroplasts (PL) of various characteristics are scattered throughout for distribution to subsequently formed daughter cells. Virus particles are discernible in some of the viroplasts during the process of cell mitosis. The cell components, vesical walls, and cell membrane are sharp, though no intact mitochondrion is to be seen in this section.

When normal chicken bone marrow is treated *in vitro* with the myeloblastosis virus, a cell population results (*24*), which is morphologically indistinguishable from that of cultures of circulating myeloblasts from the infected bird. Because of the very small number of cells available, it has not been possible effectively to sample the infected bone marrow cultures only a few days after infection. After growth sufficient to permit sampling, the viroplasts are chiefly of the form seen in older cultures. Occasionally, however, an early form is seen with the appearance of the cell in figure 2.

Adenosinetriphosphatase Activity of the Viroplast and Its Relation to Normal Bone Marrow Cells

A considerable advance in the technical aspects of the study of the myeloblast was realized when it was found, as already reported (*14*), that some of the structures designated as viroplasts in thin sections could be identified in the phase-contrast microscope and that they were thus accessible for study by cytochemical techniques. The results of investigations by these methods led to recognition of the property of certain of the viroplasts to yield a positive test for adenosinetriphosphatase activity with the Wachstein-Meisel reagent (*30*). This was of critical importance since the virus of myeloblastosis is rich in this enzyme (*37*). The presence of an enzyme in the viroplast similar to that of the virus was strongly suggestive of a relationship involved in the processes of synthesis of the virus. The possible significance of the observation was greatly heightened when it was found that certain structures, the granules, appearing in the normal cell in association with maturation of the myeloblast through the myelocyte to the adult granular white blood cell stage, likewise exhibit adenosinetriphosphatase activity. Observations by other investigators (*32, 38, 39*) have revealed a possible role of microbodies (gray bodies in the terminology used here) in the origin of mitochondria and, further, an indication (*40, 41*) that the granules of the myelocyte may be derived from mitochondria. Correlation of the results of these studies on the normal cell provides the basis for some clarification of the sites of myeloblastosis virus infection in the myeloblast, and the manner of cell response in the synthesis and disposal of the "mature" agent. Continued work with the problem has resulted in a marked refinement in the techniques for such light-microscope studies and in the unequivocal corroboration of the earlier findings, which are exemplified in the following illustrations.

The significant results of the study of bone marrow from a normal bird are illustrated in figures 8 and 9. In the positive phase-contrast picture of the living cells of figure 8, there are seen 3 myeloblasts (MB), portions of 2 myelocytes (MCL), and a heterophil (HP). The granules are revealed as dense bodies (GR) in the myelocytes and the heterophil, and none is present in the myeloblasts. In these myeloblasts, however, there are many vacuoles, the white areas, which can be identified either by supravital or fixed-preparation staining. The mitochondria show only as indefinite dark smudges. The same cells, indexed on the slide, quenched in liquid nitrogen, dehydrated, fixed with formalin vapor, and stained for adenosinetriphosphatase activity by the Wachstein-Meisel method, are shown in figure 9. It is evident that a large proportion, if not all, of the granules of the heterophil are strongly adenosinetriphosphatase-positive by this method of treatment. Similarly, many, but by no means all of the granules of the myelocytes are heavily stained. The spatial correlation between the stained granules of figure 9 and the granules seen by phase contrast in figure 8 is not perfect due to movement of the structures in the living cell during the period of observation prior to the quenching treatment in liquid nitrogen. Nevertheless, it is evident that the structures are identical. As noted before, no granules were discernible in the myeloblasts, and there is no evident positive staining for the enzyme in figure 9.

It is of much importance to emphasize the finding, which was regularly encountered, of the variation from strong to no staining of the granules of the normal myelocytes. From many studies, it seems clear that the granules seen by phase contrast by virtue of their optical properties are in variable stages of maturation. In this process, adenosinetriphosphatase is absent in the early stages of granule formation, but increases progressively to reach high concentrations in the mature granules, as can be seen by comparing the picture of the heterophil (HP) of figure 8 with HP of figure 9. Some stress should be given to the negative staining reaction of the normal myeloblasts after the process of fixation employed here. As will be discussed in a later report, improper handling of the cells during the fixation procedure results in damage to and consequent positive staining of mitochondria for adenosinetriphosphatase activity.

The findings with myeloblasts examined in this way immediately after removal from the diseased bird and those from cultures, in which the cells were not increasing, were essentially identical with those observed with the myeloblast from the normal bird. Thus, there was no evidence either of granule formation or of the presence of viroplasts identifiable either by electron- and phase-contrast microscopy or by the adenosinetriphosphatase reaction.

In striking contrast are the ultrastructural and chemical characteristics of the cells from growing cultures illustrated in figures 10 and 11. The negative phase-contrast picture of figure 10 shows 4 myeloblasts from a 61-day culture. Vacuoles (V) are seen as black circular areas. Mitochondria are numerous and scattered around the nucleus as irregular light images. A structure unique to such cells is the large bright body (PL), previously identified (*14*) as the viroplast, seen in variable numbers and sizes in thin sections in the individual myeloblasts. As seen in the same 4 cells, after fixation and treatment with Wachstein-Meisel reagent, figure 11, it is the property of these bodies, like many of the granules of the normal myelocyte and those of the heterophil, to react strongly with the adenosinetriphosphatase stain. Most of the black structures, PL of figure 11, can be identified in figure 10, particularly those in the upper regions of the upper 2 cells. Again, there has been some movement of the bodies during the period of observation. Clearly the mitochondria do not stain, and the vacuoles, which have also shifted position, are open areas.

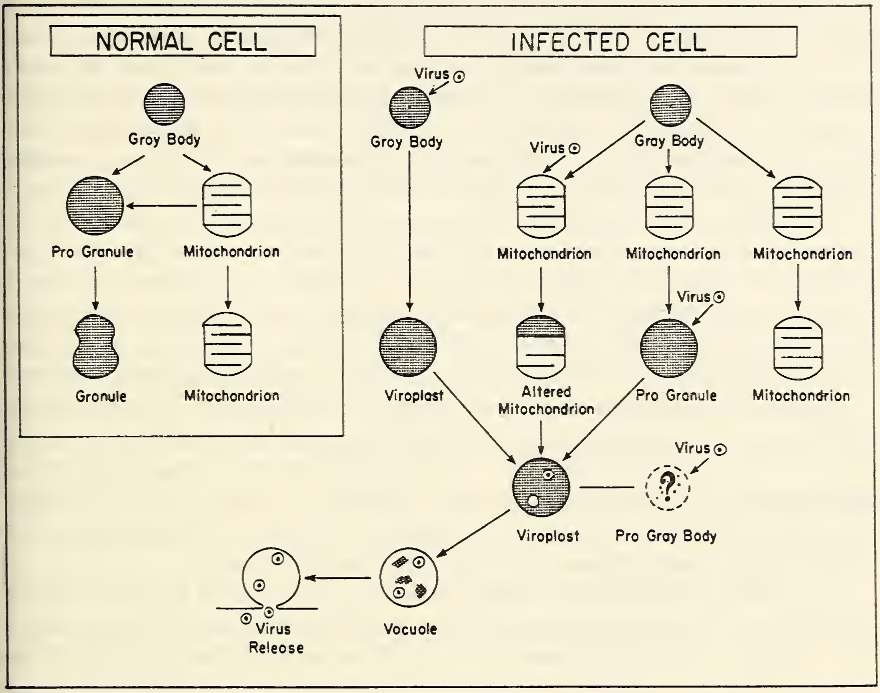
A characteristic of the viroplasts not clearly shown in this series is, as in the granules of the myelocytes, the variation in degree of staining reaction of the structures identified by phase contrast. Thus, in this instance, not all the dense bodies of figure 11, especially those of the cell at the lower right, are clearly discerned in the phase-contrast picture of figure 10, due in part to movement of the structures and to change in cell contour while the myeloblast was in the living state. Nevertheless, it has been definitely established that, although many viroplasts possess the physical properties suitable for visualization in phase contrast, their chemical attributes differ broadly with respect to the content of the enzyme. This corresponds well to the findings in thin sections, which show a wide variation in the content of the viroplast of material staining with osmium tetroxide.

Studies on Broken Cell Preparations

In earlier studies, it was found (*34*) that when myeloblasts from non-growing cultures were washed with saline and disrupted by freezing and thawing, a high adenosinetriphosphatase activity was found in the homogenate. When cell-free homogenates exhibiting high enzymatic activity were injected into chickens, myeloblastosis was not induced, though the amount of enzyme activity was 10 times more than that necessary to have caused infection if it had been associated with the typical virus particle. At that time there was no information as to the possible state of the enzyme. The results of electron and light microscopy obtained later, however, suggested that most, if not all, was present in the walled structures, gray bodies, or viroplasts. To test this possibility, myeloblasts from growing cultures were disrupted and the homogenate was fractionated by centrifugation at different speeds. By this means it was observed that the specific enzymatic activity per gram of protein was highest in the fraction coming down at speeds used for the sedimentation of mitochondria. The value observed was related to the characteristics of the cells studied, but it was always much higher than the adenosinetriphosphatase activity of normal mitochondria *(42, 43)*.

DISCUSSION

The results of the present investigations, together with those of related studies, are of principal significance in their bearing on the clarification of certain limited aspects of host cell response to the virus of avian myeloblastosis. For purposes of discussion, cell response, in the sense intended, may be regarded as the development (1) of structures physically and chemically identifiable as specific to the intracellular synthesis and subsequent elimination of the virus from the cell and (2) of processes inimical to the progressive maturation of the myeloblast in the normal pattern but compatible with continued and unrestrained growth, which is the major attribute of malignancy. Interpretation of the findings may be aided somewhat by consideration of some characteristics of the normal myeloblast and the extrapolation of the concept of the nature and potentialities of the uninfected cell to the explanation of the behavior of the myeloblast after invasion by the virus. Actually, the cell of primary susceptibility to the agent has not been identified. Inoculation of virus into the intact chicken results in the occurrence of the agent in reticular cells of the spleen and bone marrow, macrophages, and endothelial cells in the liver as discerned in thin sections (*44*, *45*), and, later in the disease, of other cells or tissues (*46*). Myeloblastic leukemia, *per se*, however, is manifested as a malignant disease only by the overgrowth of myeloblasts. This could result by direct infection of the myeloblast in its most primitive stage before the onset of the maturative processes, but the possibility must also be considered that primary infection may take place in the precursor of the myeloblast and that the virus is passed to the derivative daughter myeloblasts. The studies on infection of normal bone marrow *in vitro* (*24*) provide no unequivocal clarification of this question, since the tissue studied probably represented not only myeloblasts but other bone marrow cells as well. It must be recognized, nevertheless, that once myeloblasts are invaded by the virus, they become the representative proliferating cell, and, in consequence, the following discussion is based on the assumption that the myeloblast is the cell of primary response to the agent. The principal points for discussion are illustrated in the diagram of text-figure 1, which is concerned with those organelles thus far known or thought to be involved in myeloblast response to the myeloblastosis virus.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TEXT-FIGURE 1.—Diagrammatic representation of potential derivatives of gray bodies (microbodies) in the normal myeloblast and in myeloblasts in association with the virus of avian myeloblastosis.

The myeloblast, as the term implies, is not an end-form cell but simply a primitive element transitional between the precursor hematopoietic tissue and the mature polymorphonuclear leukocytes of the circulating blood. As such, the cell may multiply under normal conditions, as shown by mitotic figures in stained preparations of bone marrow, or begin maturation into the myelocytic series marked by the appearance and progressive increase in the number of granules. These structures, which appear to be of major significance for the present work, are of uncertain origin, but some evidence indicates that they may arise from mitochondria (*40*, *41*). In turn mitochondria have been thought to be derived (*32*, *38*, *39*), at least in part, from microbodies, or as designated here, gray bodies. It may be, also, that mitochondria may arise by segmentation of preexisting mitochondria. At the left of text-figure 1, these possible sequences have been indicated diagrammatically.

There is no reason to suppose that the principles of potential organelle derivation differ in the infected cell from those of the normal myeloblast. A major difficulty in the interpretation of the nature of cell response resides in the uncertainty of distinguishing the primary site of virus activity. At the right of text-figure 1 are indicated 4 possibilities, for each of which there exists an experimental basis.

1. The best evidence of the site of virus involvement is that concerned with the gray body. This is the structure present in the myeloblasts in the circulating blood of the diseased chicken and the structure that exhibits increase in number and size during the first hours when the cells are transferred to tissue culture. The structures transitional from the gray body to the various forms of viroplasts and, finally, to vacuoles containing virus particles, as indicated in the first sequence in the diagram to the right, can be followed clearly in thin sections of cultured cells. In this cycle it would appear that the gray body is one primary site of infection. There is no information as to the nature of the virus substance which invades the cell, that is, whether it is the whole virus or bare nucleic acid, as known for other agents (*47*). In the diagram the use of symbols resembling whole virus particles is not meant to indicate a specific concept.
2. In the second sequence it is suggested that the mitochondrion, during the process of or immediately after derivation from the gray body (microbody), may be invaded by the virus particle or the infectious moiety of the agent. For this there is considerable evidence in the frequent occurrence of structures with the appearance of residual cristae apparently in the course of alteration under the influence of the virus.
3. A third potentiality is that infection may occur in the stage of transition of the mitochondrion to the granule or progranule, if we assume the validity of the hypothesis that granules may be derived from mitochondria (*40, 41*). There is no direct evidence for this, but it is reasonable to consider a connection on the basis of the cytochemical findings, which show that both viroplasts and normal granules are rich in adenosinetriphosphatase.
4. Finally, there is some evidence to suggest that the infecting virus component may become integrated with undefined cell substance (progray body) which subsequently becomes segregated and walled and exhibits in thin sections the characteristics of the gray body or viroplast.

Despite the obscurity of the initial organelle involvement, the subsequent course of the process is clear in the progression of forms from the viroplast to the vacuole, as already described. It is of much interest to find that some aspects of the progression resemble an expression of altered potentialities with which the myeloblast was initially endowed. The findings strongly suggest that the virus enters and multiplies in structures which are the precursors of the granule. In the process the organelle retains, in part, the capacity to synthesize adenosinetriphosphatase but is subsequently destroyed and, with disintegration, is converted to the vacuole, a structure basically the normal eliminative organelle of the phagocytic cell of which the myeloblast is the precursor.

Another aberration of the natural potential of the cell is the capacity of the infected myeloblast for continued proliferation. Myeloblasts of normal bone marrow succumb after brief intervals in tissue culture (*24*), but the virus-associated cell proliferates indefinitely at exponential growth rates under the same conditions (*23*). The mechanism by which the virus confers this property on the cell is not known. There has been no evidence whatever that the nucleus is directly affected. In effect, it appears to be the influence of the agent to block all maturative processes, and for practical purposes, the cell continues to express its primitive capacity to multiply. On one point there is no doubt. It is clear that the processes of virus multiplication do not interfere with those of cell reproduction, which are carried through in an adequate growth medium with the derivation of daughter cells like the parent, each with its complement of organelles including the virus-associated structures. Seemingly, the cell is able to maintain the agent essentially outside the cytoplasm in well-walled structures. There has been no evidence of intracellular rupture of virus-containing organelles, and virus particles are not seen free in the cytoplasm. Except in rare instances, in figure 6, the virus, although multiplying rapidly as indicated by the rate of release in culture (*23*), does not accumulate in the myeloblast. This is in great contrast to the findings with reticular cells and macrophages in the diseased chicken which may contain large amounts of the agent.

In its various expressions of neoplastic response to the etiologic virus, the myeloblast of avian leukemia is thus far unique. This is evident in the well-defined specific ultrastructures which clearly indicate that the processes of cell response are organized and incorporated, for the most part, in structures and mechanisms normal to the myeloblast. As already mentioned, this does not occur in other cells invaded by the agent in the diseased chicken. That the essence of response is related to the characteristics of the myeloblast as such, rather than to a peculiar influence of the virus, is indicated by the radically different response of the erythroblast in avian erythroblastic leukemia, to be considered in another report (*48*). Although the virus of erythroblastosis is immunologically closely related to the agent of myeloblastosis (*22*), the response and behavior of the erythroblast from the diseased bird is wholly different from that of the myeloblast. A large part of this must be related to the fundamental differences in the potentials of the two host cells. It may be noted in passing that the findings with no other virus tumor cell resemble those with the myeloblast with respect to the specificity and clarity of structural involvement.

The presence of adenosinetriphosphatase in the granules of the maturing myelocytes and in those of the heterophils may well be related to the occurrence of this enzyme in the myeloblastosis virus. It seems entirely likely that the viroplasts are related in their derivation to the granules and their precursors. This suggests that the adenosinetriphosphatase may be the product of a normal cell process and that, in its development in a medium rich in the enzyme, the virus incorporates the material into its substance. It is notable that an analogous incorporation of chick-tissue antigen has already been recognized (*49*). Thus, it is possible that the presence of the enzyme in the virus is specific only in relation to the peculiarities of the medium in which it is synthesized. The antigenically related virus of erythroblastosis (*22*) contains none of the enzyme (*50*) nor does the cell, the erythroblast in which it is synthesized, contain adenosinetriphosphatase-rich organelles.

In summary: Further studies have been made of the ultrastructural characteristics of the myeloblasts of avian myeloblastosis. Cells were examined immediately after removal from the diseased bird, and the progressive structural changes occurring quickly in the cells in tissue culture were followed. As in earlier work, there was little evidence of difference, in electron micrographs of thin sections, between the structure of the myeloblast in the circulating blood and that of cells identified as myeloblasts in normal bone marrow. Within 2 hours of culture, under proper conditions, however, there was observed the development of many highly osmophilic spheroidal bodies, viroplasts, which have been identified as sites of infection and synthesis of the virus. During the first 4 to 6 days these structures exhibited characteristics which differed from those of cells longer in culture. After this period, there was seen, with considerable uniformity, the characteristic sequence of structures which varied from osmophilic bodies of homogeneously distributed internal substance to others which contained virus particles in small numbers and lesser amounts of material, finally, to vacuoles regarded as the organelle eliminating the agent from the cell.

Techniques for fixation of the cells for cytochemical study by light microscopy have been greatly refined. Additional studies have been made to identify organelles containing adenosinetriphosphatase in normal myeloblasts and their derivatives in the nondiseased bird and in the virus-associated myeloblasts from the bird and from tissue culture. Earlier observations of the enzyme in the granules of normal myelocytes and of the viroplasts of the diseased cells have been confirmed and extended. With the additional information, it has been possible to perceive possible cell-virus relationships by means of which the cell becomes involved with the virus and evidence of the contribution of normal cell processes to the multiplication of the agent. These have been indicated diagrammatically as a summary of the findings and of the hypotheses which have evolved from the studies thus far made.

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PLATE 35

FIGURE 1.—Myeloblast from circulating blood of diseased bird, washed in culture fluid in preparation for culture. Swollen appearance of mitochondria (M) and membrane around nucleus (N) is rather common in circulating cells and may be increased by centrifugal washing of these cells in preparation for culture *in vitro*. Structures marked (V) may be mitochondria which have lost their cristae by fragmentation. There are several small gray bodies (GB) and a well-defined Golgi zone (G). Relatively small size of nucleus is probably due to plane of sectioning. × 29,000

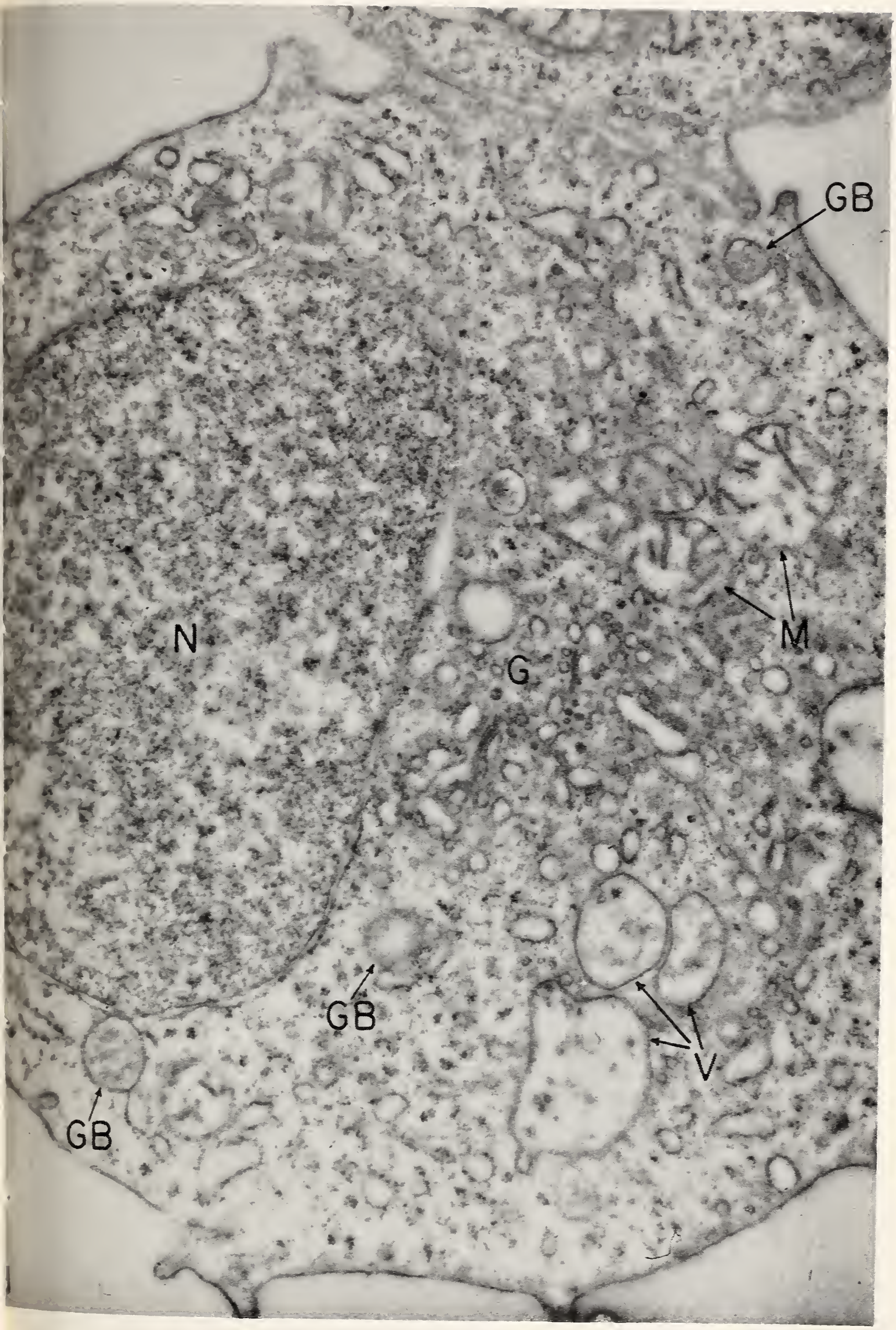
GB
M
N
G
GB
V
GB

Plate 36

Figure 2.—Myeloblast from same source as that of figure 1 after 2 hours in tissue culture in a growth-supporting medium. Mitochondria (M) are somewhat swollen, and two (AM) appear to be undergoing internal disorganization. Viroplasts (PL) occur frequently in sections of these cells. A virus particle (VP) lies outside cell. Structure of nucleus is shown at (N). × 48,000

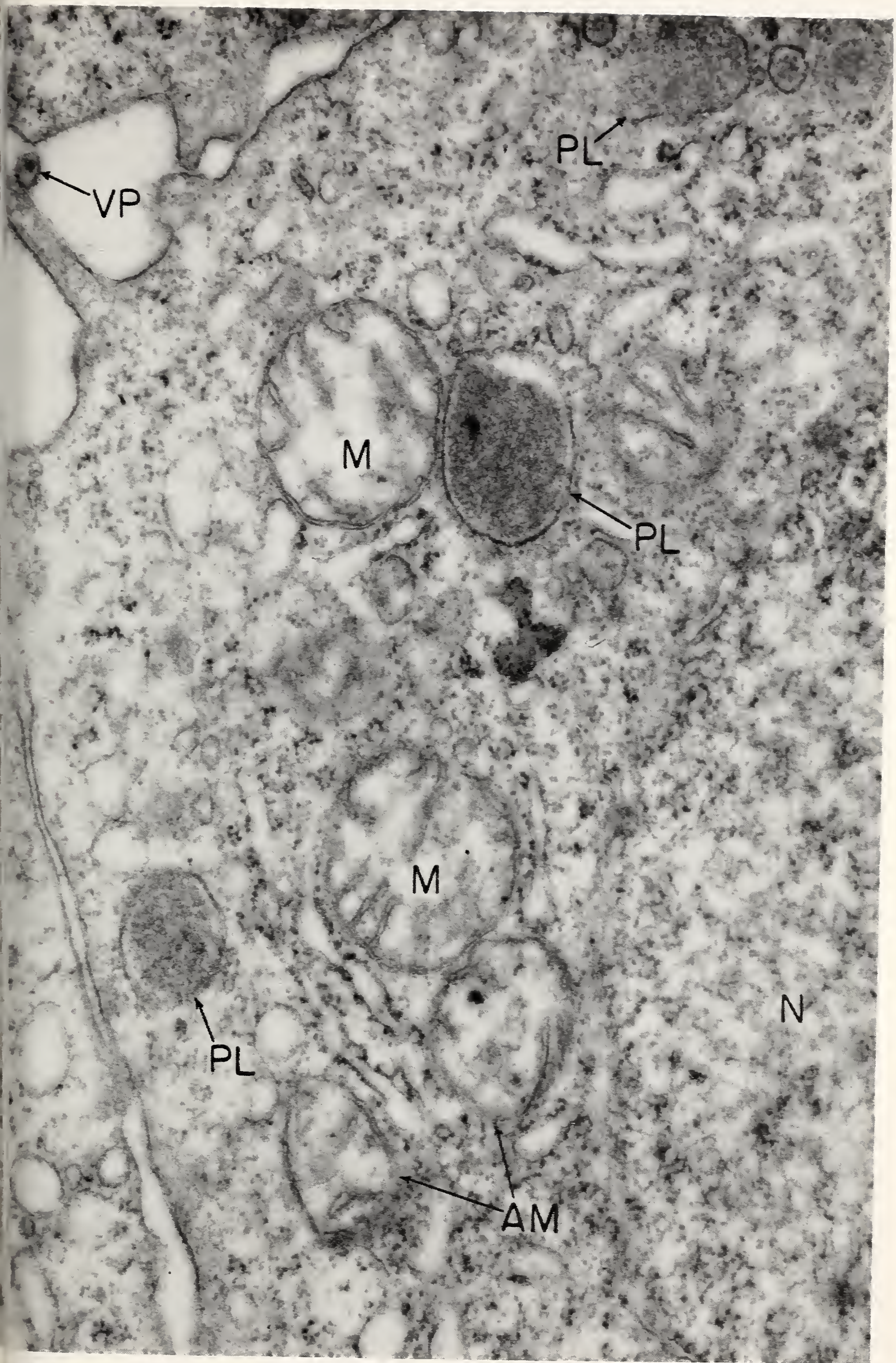
PL
VP
M
PL
M
N
PL
AM

PLATE 37

FIGURE 3.—Myeloblast from same culture as that of figure 2 after 24 hours in growth-supporting medium. Section is thicker, accounting in part, at least, for greater density of the various structures. One mitochondrion of normal appearance (M) and several which seem altered (AM) are evident. While some of the appearance of alteration may be due to plane of sectioning, this does not appear to be the whole explanation. A very large viroplast (PL) contains a virus particle (VP). Nucleus is shown at (N). × 28,000

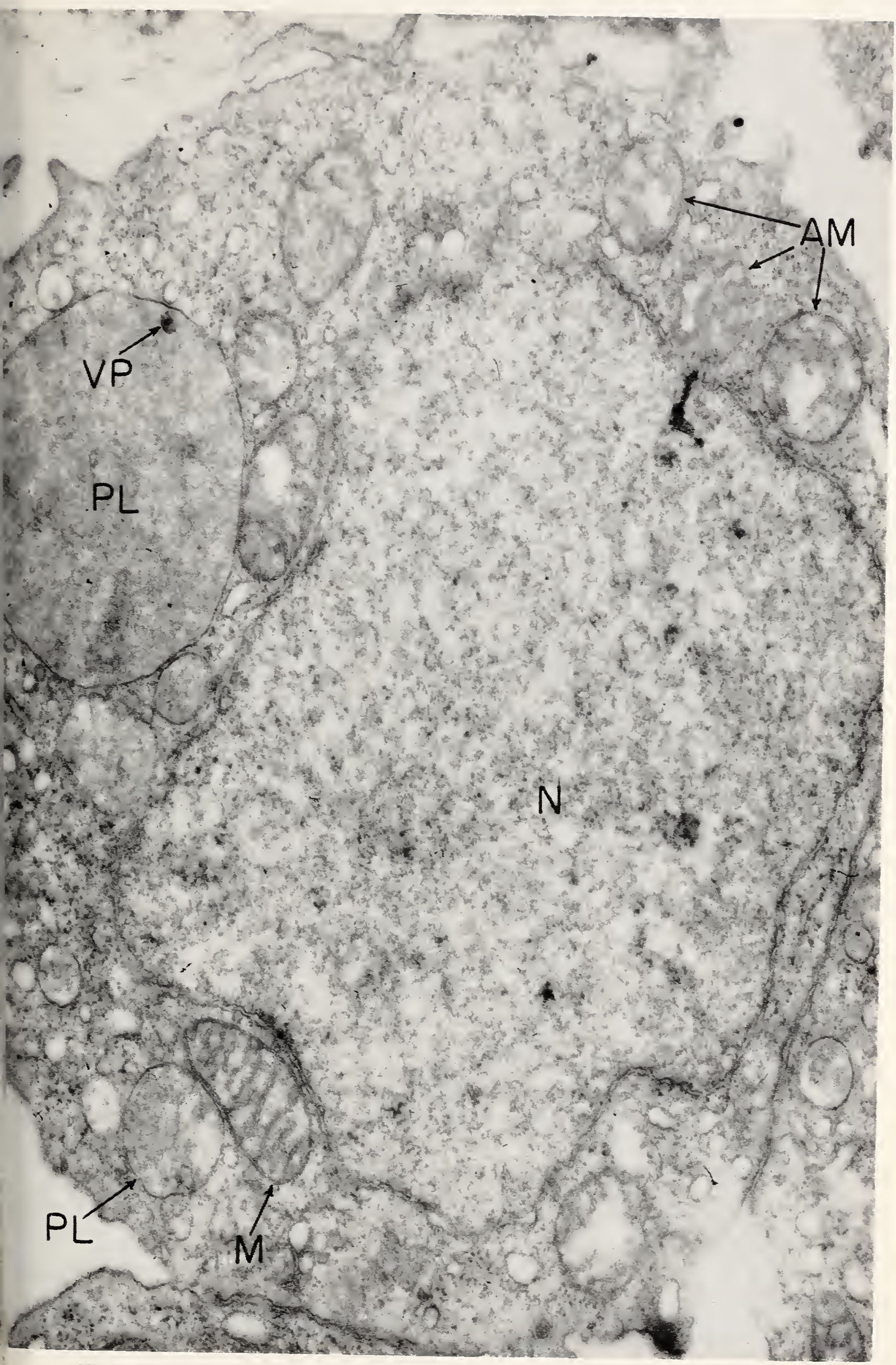
AM
VP
PL
N
PL
M

PLATE 38

FIGURE 4.—Myeloblast from growing culture 2 days old. Some of mitochondria (M) exhibit normal appearance while others show evidence of swelling. There are several viroplasts (PL) some of which contain virus particles (VP). One viroplast (PLV) contains several virus particles. Structures designated (LB) are probably lipide bodies. × 36,000

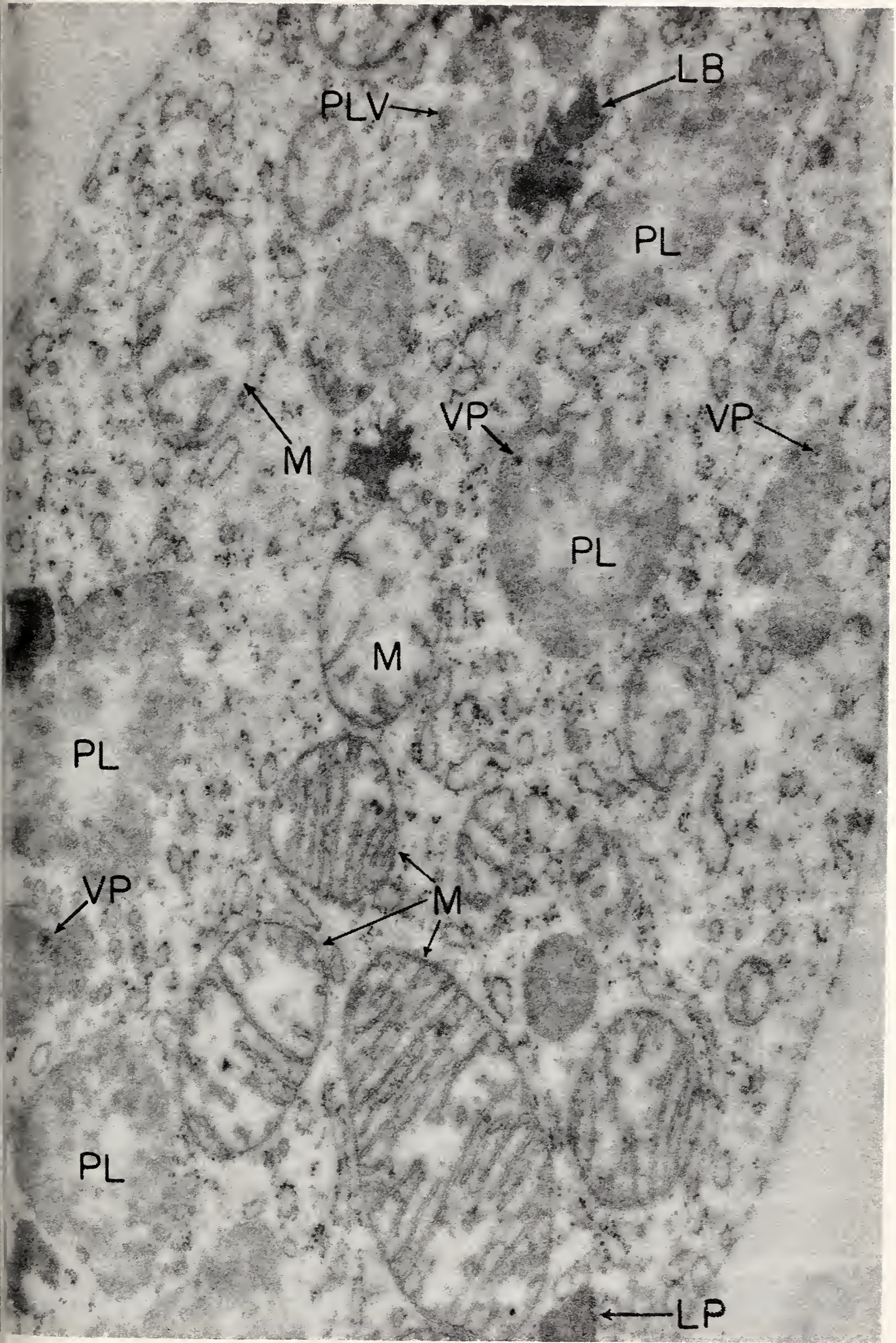
LB
PLV
PL
M
VP
VP
PL
M
PL
VP
M
PL
LP

PLATE 39

FIGURE 5. — Portions of 2 myeloblasts from a growing culture 8 days old. Several stages in postulated viroplast sequence (*see* text) are seen. These include a mitochondrion of normal features (M); a mitochondrion (AM) with fewer cristae and much amorphous material; altered mitochondria (AM) bounded by double outer walls and with only suggestions of presence of cristae; viroplasts (PL) with contents of variable homogeneity, 1 containing a virus particle (VP); and, finally, clear or almost clear vacuoles (V). × 26,000

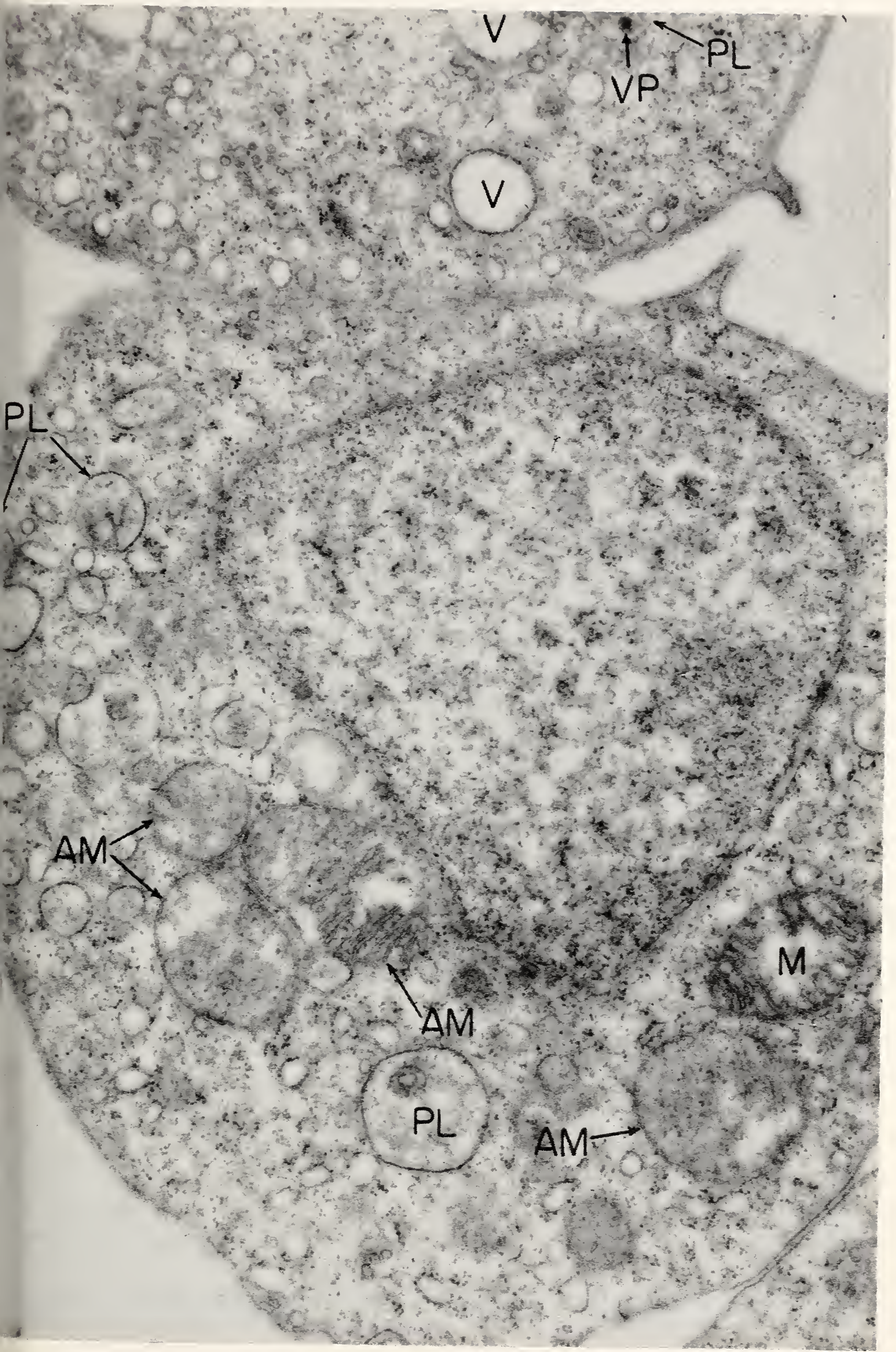
V
PL
VP
V
PL
AM
AM
M
PL
AM

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PLATE 40

FIGURE 6.—Section through edges of 3 myeloblasts (MY1, MY2, MY3) from a growing culture 12 days old showing an unusually large number of virus particles in an intracellular bounded structure (IB) at periphery of cell (MY2). A limiting membrane (LM) can be seen about this structure and lying within cell membrane (CM). Many other virus particles (VP) lie between cells. Two mitochondria (M) are also present. × 61,000

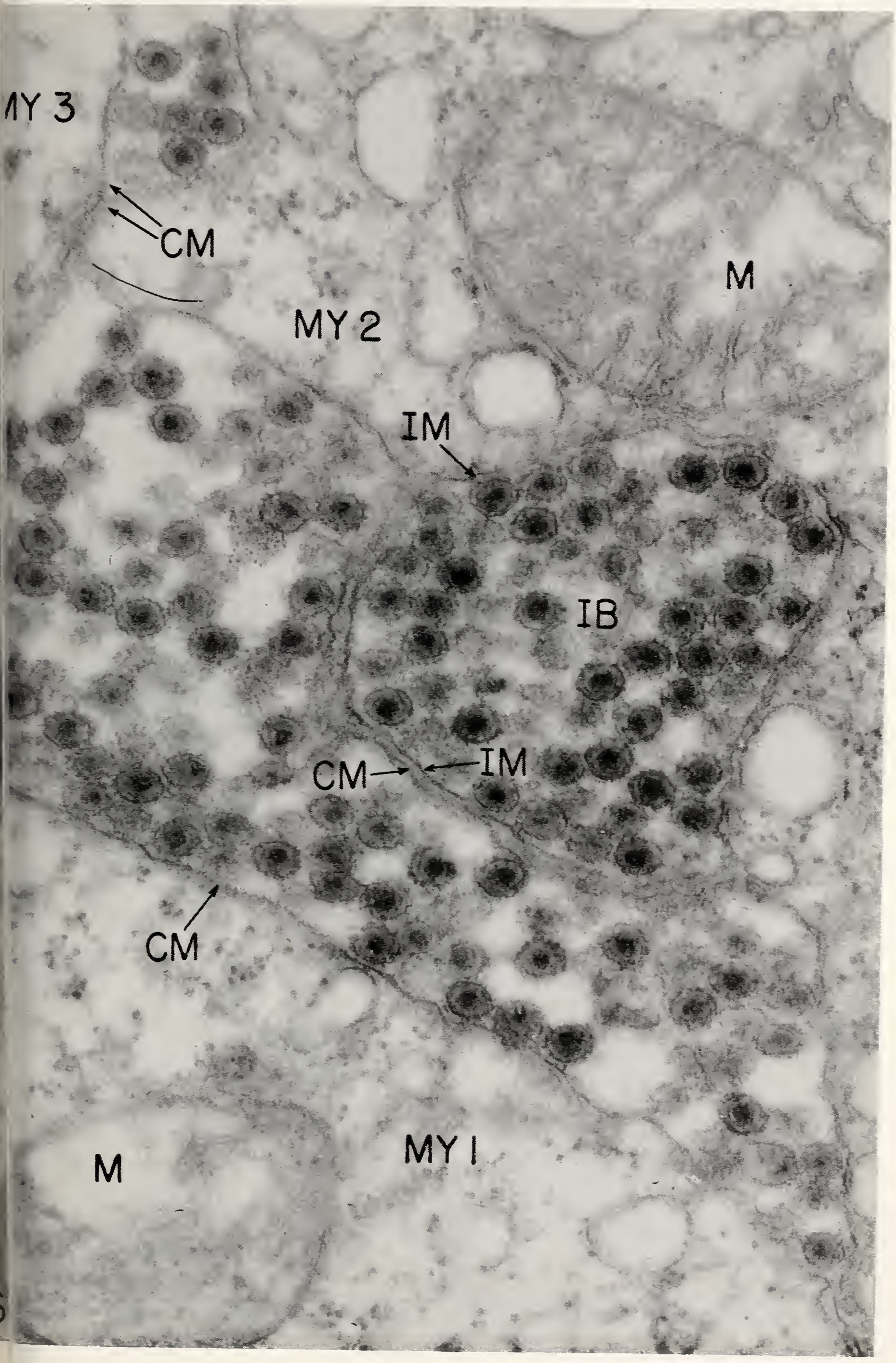
MY 3
CM
MY 2
M
IM
IB
CM
IM
CM
M
MY I

PLATE 41

FIGURE 7.—Mitotic figure of myeloblast from growing culture 2 days old. The spindle fibers (SF) extend from centrioles (C) toward chromosomes (CH). *Note* presence in cell of several typical early-form viroplasts (PL), 1 of which contains a virus particle (VP). × 19,000

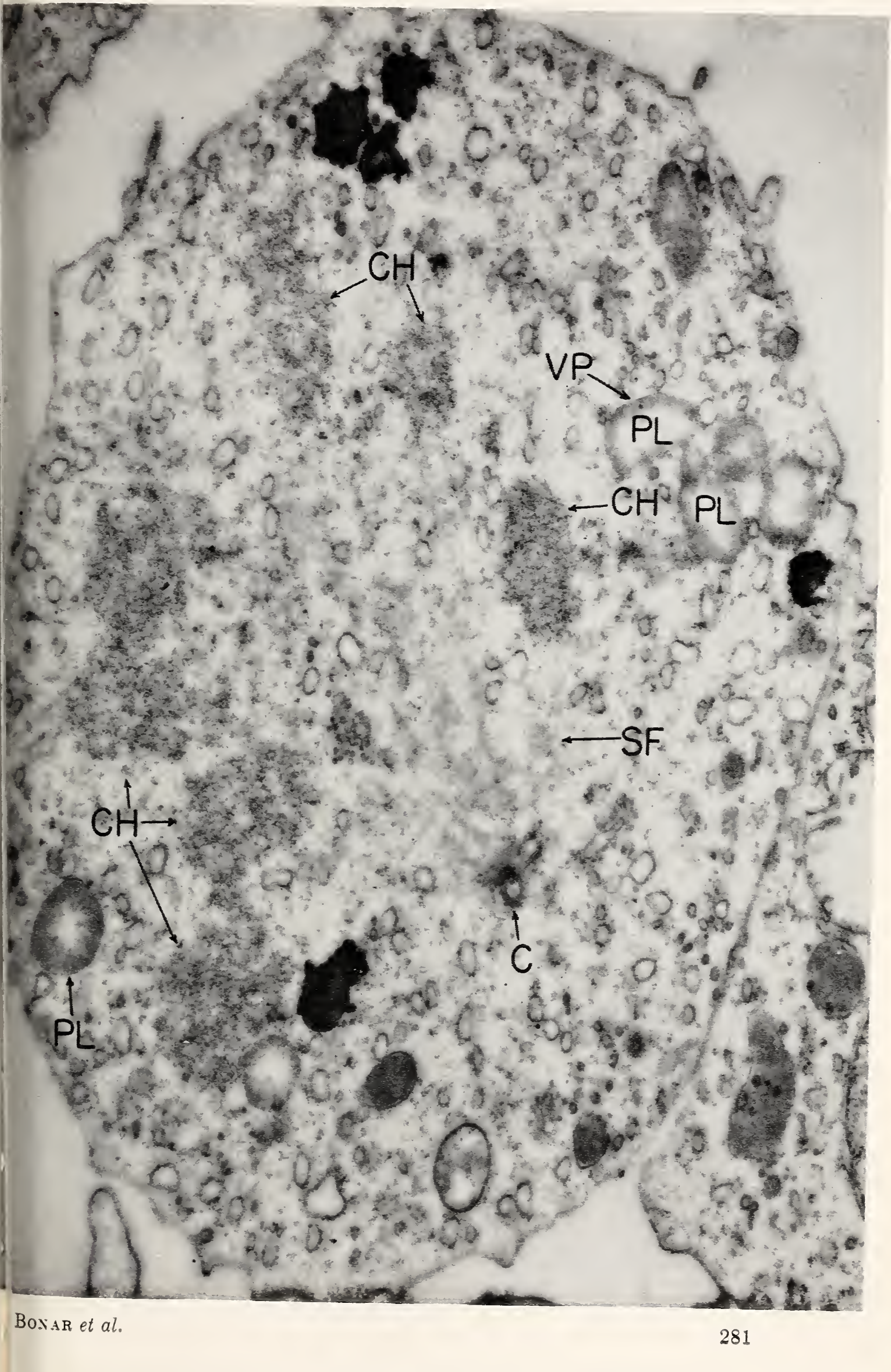
CH
VP
PL
CH
PL
SF
CH
C
PL

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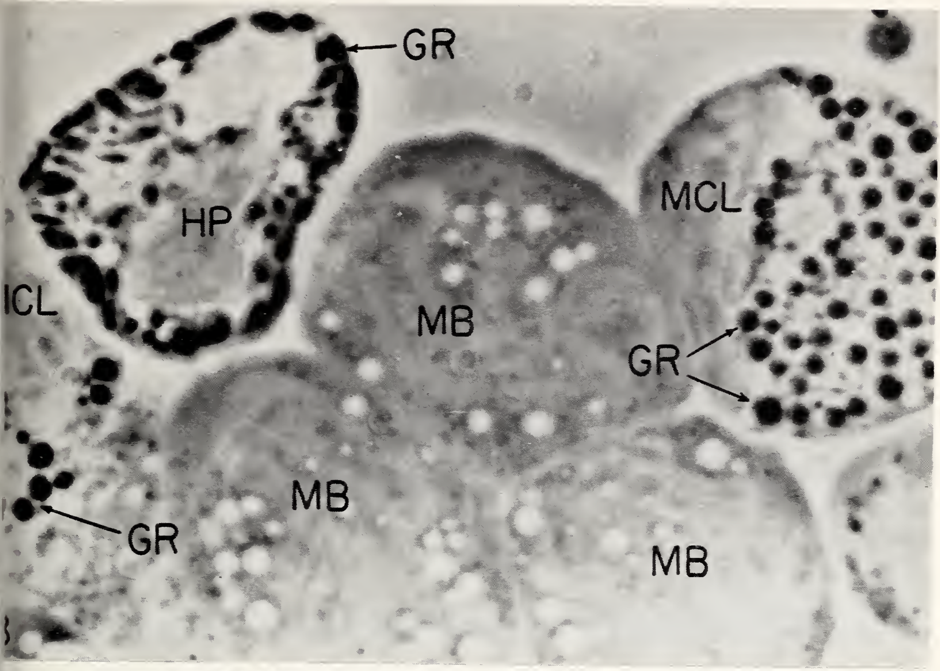
Plate 42

Figure 8.—Phase-contrast photograph of living cells from bone marrow of normal (uninfected) chicken. The respective cells were identified as a heterophil (HP); 2 myelocytes (MCL); and 3 myeloblasts (MB). Black structures (G) represent granules and white forms (V) are vacuoles. × 3,200

Figure 9.—Same cells as those of figure 8 after freezing, dehydration, fixation, and treatment with Wachstein-Meisel reagent for staining of adenosinetriphosphatase (*see* text). Symbols are same as those of figure 8. There is dense staining of some, but not all, of the granules of the heterophil and of the myelocytes. × 3,200


GR
HP
ICL
MCL
MB
GR
MB
GR
MB

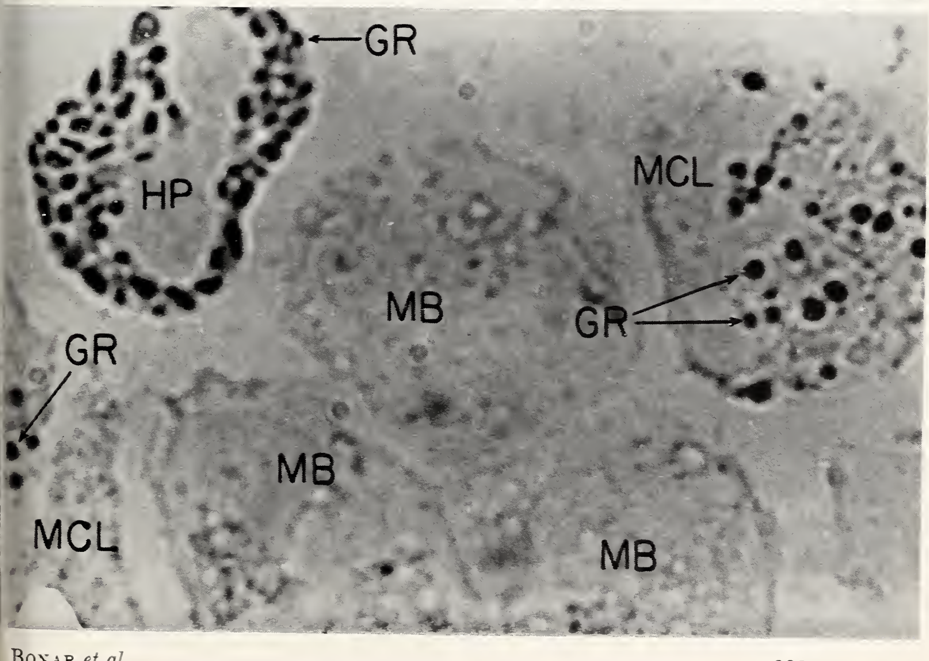
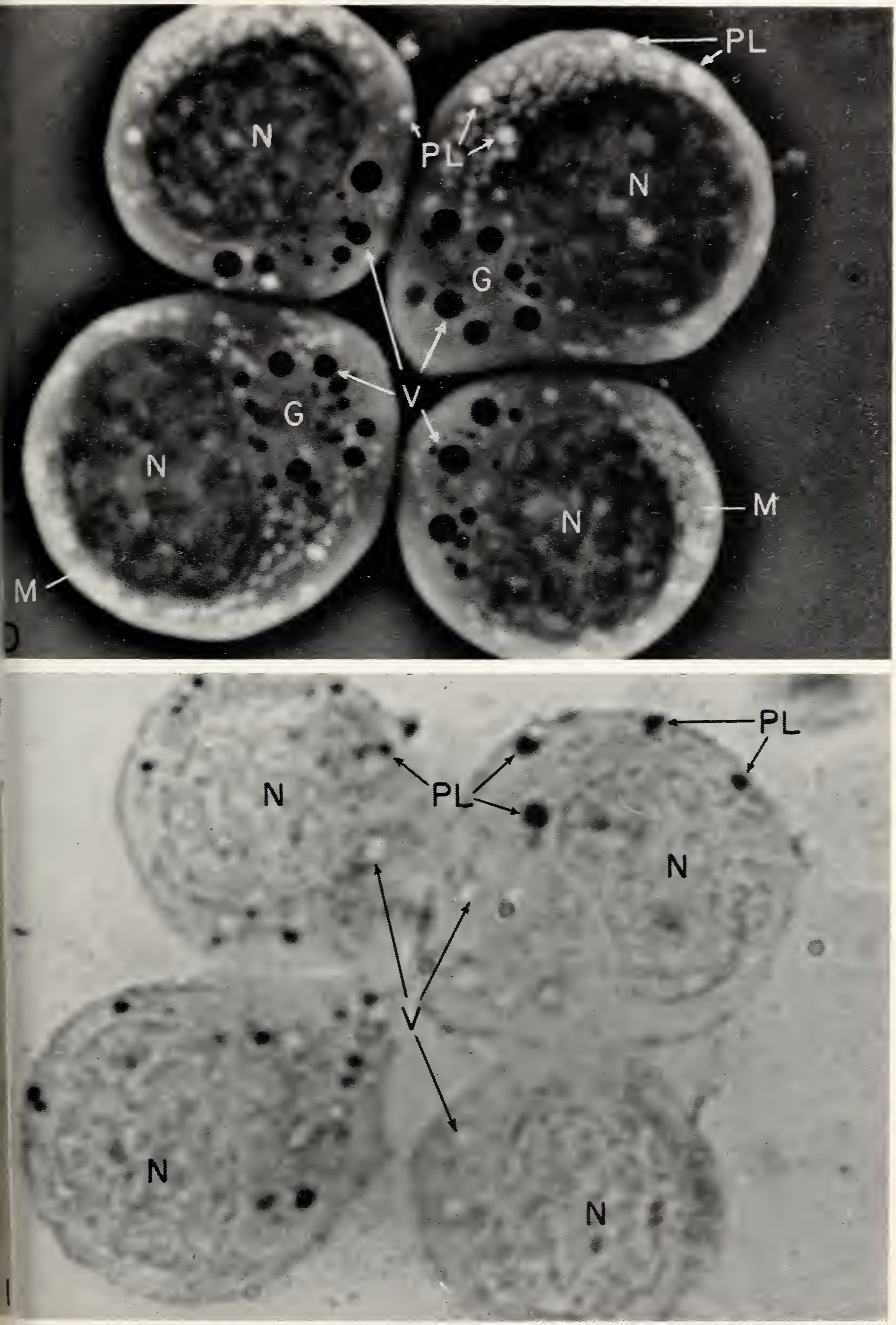

GR
HP
MCL
MB
GR
GR
MB
MCL
MB

PLATE 43

FIGURE 10.—Phase-contrast photograph of living cells from growing culture of myeloblasts 61 days after transfer from circulating blood of chicken with myeloblastosis. Nuclei (N) are large and show indications of a nuclear notch. In these regions is the Golgi zone (G) surrounded by vacuoles (V). Mitochondria (M) are distributed through the cytoplasm with few in Golgi region. Several bright structures are identified as viroplasts (PL). × 3,200

FIGURE 11.—Same cells as those in figure 10 after freezing, dehydration, fixation, and treatment with Wachstein-Meisel reagent for staining of adenosinetriphosphatase (*see* text). Symbols are the same. Many of viroplasts (PL) (bright bodies of figure 10) are heavily stained with lead. × 3,200


PL
PL
N
N
G
G
V
N
N
M
M
PL
PL
PL
N
N
V
N
N

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Discussion

Dr. Beard: Before beginning the discussion of Dr. Bonar's presentation, concerned with the discovery of the ATPase activity of the granules of normal myelocytes and granulocytes and of the general technique for the studies by light microscopy, it should be mentioned that the various sequences were devised by Dr. Mike Haddad (St. Francis Hospital, Hartford) as described in his paper now in press.

Dr. Gey (Johns Hopkins Hospital): Would it not be possible to consider that the material in the viroplasts and vacuoles was being taken up rather than being eliminated by the cells?

Dr. Bonar: From the morphologic evidence, it could not be said definitely that the material was being eliminated from the cell, because the single photographs are fixed in time. However, when the morphologic evidence is correlated with that from tissue culture, it seems that there is no other explanation. Otherwise, masses of virus should be seen inside the cell.

Dr. Gey: If that is true, then it should be possible by phase microscopic examination to see them going out. Has this been observed?

Dr. Bonar: This aspect of the problem is now under study. Of course, only the viroplasts and vacuoles could be seen in the phase microscope, not the virus particles themselves.

Dr. Francis (University of Michigan): Would you explain the function of the viroplast in the production of virus in the myeloblasts that is unlike the functions of the vacuoles Dr. Haguenau observed, in which virus was seen in Rous sarcoma cells? It seemed to be her view that the vacuoles and the particles in them were the result of pinocytosis.

Dr. Bonar: Again there arises the problem of interpreting a dynamic process from static pictures. It is possible that some of these vacuolar inclusions in the myeloblasts do arise by phagocytosis or pinocytosis of virus particles, though, personally, I think this is probably not the case.

We believe that the virus particles seen in the vacuoles in the myeloblasts are derived from the viroplasts, because there is evident the range of structures apparently involved. This begins with bodies with a dense, rather homogeneous matrix, in which one can occasionally see faint outlines of virus. From these dense bodies there is a spectrum of structures including those with distinct virus particles in a dense matrix, those which have lost part of their nonviral content, and, finally, the vacuoles which contain virus particles and little else.

While it is possible that some virus particles might have reached vacuoles by the process of phagocytosis, it seems extremely unlikely that the process could be reversed all the way to the structures with a dense matrix with only one or a few virus particles embedded in this matrix.

With respect to Dr. Haguenau's pictures, there is the same difficulty of interpretation. I suspect that our gray bodies are similar to the rather dense structures containing virus particles which she has seen in Rous sarcoma cells, and that they are probably analogous to the altered mitochondria containing virus particles which Benedetti and Bernhard have observed in erythroblastosis. We have not had the good fortune to see virus particles in a really recognizable mitochondrion in myeloblasts, though we have searched diligently. It may be, of course, that virus particles do not occur in recognizable mitochondria in myeloblastosis.

Dr. Haguenau: From Dr. Bonar's pictures I would be convinced that he is right in the assumption that these particles, though we cannot say anything morphologically, are being extruded from the myeloblasts, because he has other evidence of the process. I was impressed that he did not have (at least in the pictures he has shown and, of course, he did not choose them for that) any of this amazing pinocytosis activity that we have seen together with extraordinary pseudopod activity of Rous sarcoma cells, which I have not illustrated directly. Nevertheless, this is very striking. I am not sure that the situation with Rous sarcoma system is absolutely comparable with that of myeloblastosis.

I have the feeling, too, that these gray bodies which we have seen only rarely in Rous sarcoma cells might be increased greatly in number if we could improve our tissue-culture medium. Perhaps, then, more could be seen, and they might be found really to correspond to virus formation centers. Up to now our pictures are not absolutely comparable to yours in that respect, but this does not mean, however, that the Rous system might not be similar to myeloblastosis if we could improve our methods.

Dr. Palade (Rockefeller Institute): Dr. Bonar, how good is your evidence that what you call "small gray bodies" are the normal precursors of mitochondria?

Dr. Bonar: Our evidence on this point is negligible. We have simply observed that these structures are rather frequent in these cells. If mitochondria are involved in the viroplast sequence and normal mitochondria must be formed as replacements, it seems possible that these are precursors. We have simply taken this idea from the literature, and it is not an essential point of our argument. We are not certain that they are precursors.

Dr. Palade: According to your diagram of the normal cell, the gray body is the precursor of both mitochondria and granules. What granules do you have in mind?

Dr. Bonar: I was thinking of the granules of myelocytes and of mature granulocytes which are derived normally from myeloblasts. There have been two reports in the literature concerning the origin of these granules. In both, formation from mitochondria was suggested. This was based on electron microscope evidence.

Dr. Palade: Do you have any evidence about the enzymatic content of those granules in the mature white blood cell?

Dr. Bonar: Yes, we have, from the application of the staining technique of Wachstein and Meisel. It appears that in the mature cells ATPase activity is localized in the granules. It might be mentioned, also, that there is some difference in sensitivity to various fixation procedures between the granules of the myelocytes and those of the mature cells. Also, you may have noticed that only some of the myelocyte granules reacted, while almost all the granules of the mature cell were positive.

In the viroplasts in the infected myeloblasts, preliminary studies have been made to isolate the bodies by centrifugation of cell homogenates. It was found, in the electron microscope, that the mitochondria and the viroplasts appear together in the usual mitochondrial fraction. It has not yet been possible to get this fraction in a satisfactory state of purity, since there is always much apparently microsome material present. However, the ATPase activity of this fraction is much higher than that which has been reported for other mitochondrial material, that is, from liver and so on.

Dr. Palade: Is it not possible that the ATP is split by another phosphatase in the granule?

Dr. Bonar: Other substrates have been tested including ADP, AMP, and glycerophosphate, and a little activity was found with ADP. It is difficult to quantitate the reaction very well with these cytochemical techniques, but it appears that the reaction with ADP was less than that with ATP. With AMP and glycerophosphate, there was essentially no reaction.

However, if the cells are damaged by sufficient pressure under the coverslip to cause bursting or extrusion of processes from the cell, the mitochondria may exhibit ATPase activity.

Dr. Palade: Do you visualize, then, that the ATPase activity originally found in the gray body or in the granule is transferred, finally, to the virus particle?

Dr. Bonar: Yes, it is reasonable to suppose that, as the virus particle forms in this environment containing ATPase, it incorporates the enzyme, perhaps only incidentally. It might be noted that the virus particle also contains normal chicken tissue antigen which is probably picked up in the same way.

Dr. Palade: Finally, would it not be possible to use a tracer material visible in the electron microscope to determine whether the vesicles containing the virus particles are opening and releasing the virus particles to the medium or perhaps taking them up from the outside?

Dr. Bonar: It is possible. The plan at present is to examine the cells with phase contrast over long periods for observation of the movement of the bodies. The cells have not been tested for phagocytic activity, but this is also planned.

Dr. Rose (Columbia University): Apropos of the observation Dr. Bonar has made in connection with the possible release mechanism of viral particles from the myeloblast and also of the discussion which followed his presentation, perhaps it would be helpful to indicate that evidence obtained with another virus suggests the operation of the same sort of mechanism.

Dr. Morgan, Dr. Holden, Dr. Jones, and I reported, in the *Journal of Experimental Medicine* last fall, some observations with herpes simplex virus in which exactly this kind of mechanism was postulated as the result of the electron microscopic observations. I, for one, am convinced from Dr. Bonar's data that this mechanism is operating in the agent he has under consideration, and I have no doubt that this same mechanism probably operates in other viruses.

Do you view the formation of virus as not a destructive process but as the continuing elaboration of the agent by surviving cells? If that is the case, within any one cell, is the formation of virus a synchronized process? Or do you find within a cell, a single cell, all stages of virus formation?

Dr. Bonar: After the first period of 2 to 4 days in culture, we often find in one cell section a number of different stages of what we believe to be the process of virus production.

Dr. Dmochowski (M. D. Anderson Hospital): Dr. Bonar, have you found in the cells grown in tissue culture the formation of membranes in the cytoplasm that Dr. Haguenau has described as myelin figures and which we call membranous structures?

Dr. Bonar: No, with the possible exception that we see vacuoles or vesicles which do not contain virus particles. Of course, particles may be there, but outside the plane of sectioning. We have seen nothing resembling myelin figures or these multiple membranous components.

Dr. Dmochowski: A comment which I believe is pertinent concerns the satisfaction one feels when he observes changes in tissue culture similar to those seen in the cells of organs of chickens infected with the same type of disease. We have observed similar structures—we do not describe them as gray bodies but as osmiophilic bodies—and the presence of virus particles in mitochondria. It is pertinent, also, to point out that similar changes have been observed by us in erythroblastosis of two strains, RPL12 and the Engelbreth-Holm strain R and, too, in visceral lymphomatosis, but not in Rous sarcoma.

Therefore, I was interested to hear from Dr. Haguenau that a similar trend of events is taking place in Rous sarcoma cells, if I understood her correctly, when the cells are grown in tissue culture.

In addition, similar changes—*i.e.*, a similar developmental cycle—take place in renal adenocarcinoma induced in chickens by the myeloblastosis virus. Thus, it appears that there is some uniformity in what might be described as the developmental cycle in this group of viruses.

Dr. Bonar: It is interesting that there seem to be some developmental relationships, even as there are morphologic and serologic relationships among the virus particles themselves. But someone suggested that we are dealing with a unique cell. This is certainly true, though I suppose we must regard each cell type as unique. In the normal course of events, as the myeloblast matures, it has the capacity to form granules. We do not know yet what relationship these structures in the myeloblasts may have to the bodies of similar appearance in erythroblastosis or other chicken tumors. It is tempting to speculate that the malignancy of the myeloblast is due to the inhibition of maturation of the granules and thereby the maturation of the cell.

Dr. Beard: I would modify that somewhat—not the inhibition of the maturation of the granules, but the inhibition of the maturation process of the total cell which includes maturation and the formation of granules.

Dr. Miroff (University of North Dakota): Dr. Bonar, you mentioned that you saw lipoid bodies in these infected cells. Do you find these generally associated with infected cells?

Dr. Bonar: They are rather frequent in these cells.

Dr. Miroff: Are they more prevalent when you see more virus particles?

Dr. Bonar: Generally they are not so numerous in the rapidly growing cultures. They are more apt to be seen in the circulating cells in the diseased chicken or in cells which are not growing. This has not really been quantitated, but it is my impression that they are less common in the growing cultures.

Dr. Gemile Haddad: In some of the pictures Dr. Bonar showed, the viroplasts were vacuolated and contained virus particles. I wonder whether the particles should be considered intracellular or extracellular. This may be only of academic importance, but if they are to be regarded as extracellular, the relationship would be analogous, say, to food in the human stomach, which is extraorganismal rather than intraorganismal.

Dr. Bonar: This is a matter largely of terminology but still of interest. The question might be asked whether the cristae of the mitochondria are intra- or extracellular on the same basis. I do not know how to answer your question.

Dr. Banfield (National Institutes of Health): In the structures which you referred to as the very early gray bodies with a dense membrane and a rather granular center, there is a similar structure, almost identical, which is seen in cells infected with molluscum contagiosum. These have been described by Dourmashkin, and he has attributed their origin to mitochondria, but with what evidence, I am not aware.

There is also a tissue-culture cell, grown by Dr. Dawe, that contains many bodies identical to the gray bodies and even contains particles like those which can be called virus-like particles. This is a cell derived from a mouse lymphoma.

Dr. Scherer: Have you observed that cells containing viruses undergo mitosis?

Dr. Bonar: Yes. It was probably because of the low magnification of those two cells in mitosis that you were not able to see the virus particles. However, they did contain the typical structures which we have observed in the purified virus preparations and which have been definitely associated with infectivity.

Dr. Dalton (National Institutes of Health): In all these situations as they have been described by Dr. Haguenau and you, the respective cells have been in tissue culture. As I understand it, this whole group of avian viruses is presumably derived from a common source or have basic similarities. Yet, it has already been described that, in erythroblastosis, particles do bud out from a surface membrane. What do you think about the possibility that the tissue-culture situation may be a special one and that, under conditions in the host *in vivo*, the actual life cycle might possibly be different?

Dr. Bonar: The results with erythroblastosis really suggest the need for considerable caution in judging the quantitative importance of various aspects of virus production. Dr. Heine in our laboratory has observed that different cultures of erythroblasts may show differences in the frequency of budding in relation to other possible modes of virus formation. In the examination of hundreds of pictures of myeloblasts, there were three suggestive of the occurrence of buds. Conceivably, then, this process may occur in myeloblastosis as well.

Examination of cells in various tissues of birds with myeloblastosis has not shown budding, but the sampling problem in electron microscopy is such that we may have missed it, and there are other tissues which we have not examined.

Dr. Murray (Columbia University): Since mitochondria seem to be so important to some of our discussions, would Dr. Palade have a suggestion or a statement as to the origin, the genesis of mitochondria? Would he tell us where they come from, where they go, and whether they are self-perpetuating?

Dr. Palade: The best evidence so far available indicates that mitochondria are self-perpetuating bodies resulting from other mitochondria that preceded them. It is interesting in this respect to note that in the life of an animal cell there is no stage at which the mitochondria disappear.

Experiments, performed sometime ago by Ethel Harvey, have been interpreted as evidence for the view that mitochondria can arise *de novo* in the cytoplasmic matrix.

Dr. Harvey centrifuged *Arbacia* eggs until they broke into two halves with all the mitochondria apparently sedimented in the heavy halves. The light halves, which, by the way, contained the lipide inclusions of the eggs, developed into embryos upon fertilization, and in the cells of these embryos, mitochondria could be convincingly demonstrated. Since the cells were derived from apparently mitochondrion-free eggs, it was concluded that their chondriome arose *de novo.* But more recently, light halves of *Arbacia* eggs have been examined by electron microscopy and found to possess mitochondria closely associated with their lipide inclusions. Therefore, the original interpretation of the experiment is now in serious doubt.

So here the evidence rests for the moment. We tentatively assume that any mitochondrion derives from a preceding mitochondrion, but we should realize that the assumption is based primarily on the lack of clear evidence to the contrary.

Electron Microscopy of Swiss Mouse Leukemia Virus[1, 2]

ETIENNE DE HARVEN,[3] *M.D., and* CHARLOTTE FRIEND, *Ph. D.,*[4] *The Laboratoire de Microscopie Electronique de l'Institute d'Anatomie, Université Libre de Bruxelles, Brussels, Belgium, and the Section of Viral Oncology, Sloan-Kettering Institute for Cancer Research, New York, New York*

The virus-induced leukemia of Swiss mice is at present being studied in several laboratories, with different approaches. As part of this team work, the electron microscopists can provide information dealing with the control of biological experiments or with the purely morphologic description of the virus. Since the former findings on this leukemia have been reported (*1–5*), this paper will deal only with electron microscope observations on material prepared with the techniques previously described (*1, 2*).

IDENTIFICATION, FREQUENCY, AND LOCALIZATION OF THE VIRUS

The virus particle observed in the leukemic material is round or ovoid in shape and has a mean diameter of 870 Å. The main features of this virus are the concentric spherical membranes, the outer and the inner virus shells. Ultrathin sections of pellets prepared by ultracentrifugation of the leukemic filtrates have revealed the same type of particle identified in the liver, spleen, and bone marrow of the leukemic mice. The observations made in these tissues have been entirely superimposable up to now as far as virus-host cell relationships are concerned.

The specimens were fixed at different stages of the leukemic process from the 21st to the 78th day after inoculation with a cell-free leukemic filtrate. The virus was found in approximately 40 percent of the specimens taken from mice treated with leukemic filtrates. In this group of animals no preliminary study of the blood was made to indicate how generalized the disease was at the time the animals were killed. More recently, when specimens were taken only if the blood smear showed

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[1] Presented at a Symposium on Phenomena of the Tumor Viruses, New York, N.Y., March 25 and 26, 1960.

[2] This investigation was supported in part by a research grant from the National Cancer Institute, National Institutes of Health, Public Health Service.

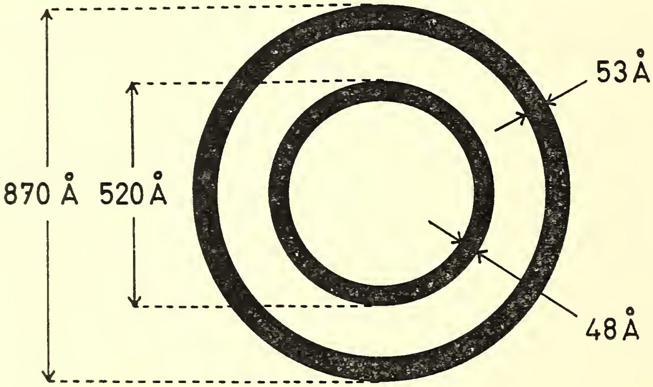
[3] Associé du Fonds National de la Recherche Scientifique (Belgium), Laboratoire d'Anatomie Pathologique, Université Libre de Bruxelles, Brussels, Belgium.

[4] The technical assistance of Miss Pauline de Looper, Mrs. Joyce B. Borelli (aided by a grant from the Sloan Foundation), Mrs. Mona Brandreth, Miss Violet Darchun, Mr. Gilbert Holland, and Miss Carol Mays is gratefully acknowledged.

marked leukocytosis with many primitive cells and erythroblasts, particles appeared to be present in a considerably higher number of cases. Unfortunately, even in such material virus may be seen only in small numbers. This is possibly due to the choice of material, for it is conceivable that the virus reservoirs are located in other tissues or observable at different stages of the leukemia. Up to now, however, there is no clear relationship between the presence of the virus in the tissues and the stage of the leukemic process. All the viruses were found outside cells, in the intercellular spaces, or in intimate contact with the cell membranes. The virus was never observed in the cytoplasmic matrix, in the cytoplasmic organelles, or within nuclei.

THE FINE STRUCTURE OF THE VIRUS

High-magnification studies of the virus were facilitated by the lead hydroxide technique described by Watson (*6*). With this procedure a supplementary membrane of the virus located between the outer and the inner virus shells and measuring approximately 60 Å in thickness was demonstrable. The mean dimensions of the virus are shown in the schematic drawing in text-figure 1.



Text-figure 1.—This schematic drawing illustrates only the mean dimensions of the shells of the Swiss mouse leukemia virus. These dimensions are subject to wide individual variations. No attempt has been made to indicate the precise distribution of the electron-dense material in this virus-membrane system and the high density of the inner virus shell observable after lead hydroxide staining.

The density of the virus material is usually higher in the peripheral portion between the outer and the inner shells than in the central portion of the virus. Such a virus may be related to the virus type "A" described by Bernhard (*7*). Occasionally, however, the reverse situation is observed, the center presenting the maximum electron density corresponding to virus type "C" described by Bernhard. In this case the dense material present in the center of the particle is usually irregularly distributed, without any suggestion of a twisted filament. In a few instances, however, a dense nucleoid is observed. The intermediate membrane is not always recognizable, and it is not yet clear whether it should be considered a definite component of the virus or whether it corresponds to the condensation of the material between the outer and the inner virus shells. The lead hydroxide technique does not seem to modify the dimensions of virus components and greatly increases the contrast of the virus, especially at the level of the inner shell which presents a maximum affinity for lead hydroxide. This remarkable affinity of the inner shell for the lead hydroxide is comparable in intensity to the affinity presented for the same salt by the cytoplasmic ribonucleoprotein (RNP) granules.

RELATIONSHIP OF VIRUS TO CELL MEMBRANES AND THE HYPOTHESIS OF BUDDING OF VIRUS FROM THE MEMBRANES

The frequency of the relationship between the viruses and cell membranes has been already demonstrated in the study of the virus fine structure. Further it has been possible to observe various forms of incomplete viruses appearing as a differentiation of the cell membrane itself. Since it is dangerous to describe dynamic processes from observations made on fixed tissues, it is fully recognized that the sequence of figures 1, 2, 3, and 4, in that order, is hypothetical. Nevertheless, by analogy with equivalent processes already known to occur in the development of other viruses, it is reasonable to consider those pictures as describing the different steps in maturation. The budding process seems to be the transitional phase of the virus cycle between the proviral stage and the mature extracellular infective particle. The rupture of the narrow pedicle probably liberates the mature virus into the intercellular spaces.

Two points more specifically related to present observations should be stressed: (1) Obviously the inner virus shell differentiates at the level of the cell membrane and does not correspond to a preformed vesicle coming from another area of the cytoplasm. (2) The virus, while budding from the cell surface, is definitely a type "A" particle with its maximum density at the periphery and not in the center. This indicates that the "A" particle is a recently formed virus and that the type "C" is possibly further along in development.

THE PARTICULAR CASE OF THE MEGAKARYOCYTES

Light microscope studies of leukemic tissues do not indicate any pathological alterations of megakaryocytes. These giant cells appear normal in structure and in frequency and apparently do not participate in the leukemic proliferation. Nevertheless, electron microscopy demonstrates the viruses in a suprisingly large number of megakaryocytes (fig. 5), the cells being frequently overloaded with viruses morphologically identical to those at the surface of the leukemic cells. The only particularity of the viruses observed is megakaryocytes is the frequent occurrence of dumbbell or chainlet profiles corresponding to the continuity of the outer virus membrane of several viruses.

This observation probably means that the virus may multiply in a cell that is not necessarily leukemic. In the megakaryocyte, the viruses are not found at the periphery of the cell, but within cytoplasmic vacuoles. It is believed, however, that these vacuoles are derived from the endoplasmic reticulum, and that consequently their membranes could be related to the cell membranes. These vacuoles are therefore equivalent to extracellular spaces, and the particular case of megakaryocytes is not in contradiction to the statement that Swiss mouse leukemia virus is always found outside the cell or at the cell surface.

The hypothesis that the localization of the viruses within megakaryocytes might be the result of the phagocytic activity of these cells does not hold for two reasons: (1) Classical light microscopy studies have demonstrated the absence of phagocytic activity in megakaryocytes (*8*), and (2) incomplete viruses were observed budding from the membranes limiting the cytoplasmic vacuoles of the megakaryocyte, which showed once more how closely the cell membrane and the endoplasmic reticulum membranes are related.

PROBLEM OF INTRACYTOPLASMIC PRECURSORS

Despite the fact that the viruses are budding at the surface of leukemic cells, it has not thus far been possible to identify positively any earlier step in the virus cycle. Before the tiny bulge at the cell membranes is demonstrable, there must be another part of the virus cycle not observable with our present morphologic techniques. In leukemic cells, the Golgi zone is frequently large and presents numerous vacuoles, but no relationship between these Golgi structures and the earlier steps of virus development has been established. In two cases, however, a large vesicle beneath a budding virus was seen. Such a rare observation is, of course, of questionable value, but it is noteworthy that a similar phenomenon was seen by Benedetti and Bernhard (*9*) in their study of chicken leukosis.

PRELIMINARY AND NEGATIVE OBSERVATION ON RIBONUCLEASE (RNase) DIGESTION

Lead hydroxide staining does not seem to have a cytochemical specificity but, as already mentioned, it has a maximum affinity for the inner virus shell comparable in intensity to that for RNP granules. In an attempt to demonstrate whether or not ribonucleic acid is present in the virus, and with the observations of Epstein (*10*) in mind, RNase digestion was used on material fixed with permanganate and embedded in Araldite. So far the results have been entirely negative. Although leukemic specimens embedded in Araldite are useful for low-magnification studies, it is known that the recognition of RNP granules in such permanganate-fixed material is difficult. Unfortunately, this was also true for the virus. It was therefore necessary to devise another technique: After the specimen had been fixed in buffered formalin, it was subjected to RNase digestion. It was then fixed a second time in osmium tetroxide before being embedded in methacrylate.

After this procedure the RNP granules seemed to disintegrate, even though the fixation was poor. The viruses, however, appeared resistant to RNase digestion under these experimental conditions and retained a normal structure. This may be related to the nature of the outer virus shell and is being investigated further.

SOME PRELIMINARY RESULTS OBTAINED IN OTHER LYMPHATIC TUMORS

In the past, many attempts to transplant so-called spontaneous lymphatic neoplasms, which arise in older Swiss mice, were unsuccessful. However, within the last year, at least 5 tumors have been found that are readily transplantable to mice of this randombred strain; 2 of these 5 have now been examined under the electron microscope. Particles have been found in both, and the other 3 remain under study.

In view of this phenomenon, the observations in one of these tumors are presented: In a group of Swiss mice inoculated with leukemia-virus nucleic acid, a lymphoblastic lymphoma arose in 1 of the mice 5 months after treatment. This lymphoma is now in its tenth serial generation and is being followed throughout by electron microscopy. The material presented here illustrates the findings obtained in specimens from the first three generations.

The electron microscope study of this transplantable lymphoma shows an extremely large number of intracytoplasmic doughnut-shaped particles (figs. 6, 7, and 8) that do not resemble the leukemia virus. These particles, the diameter of which is about 800 Å, form large inclusion bodies frequently in the Golgi zone and sometimes in contact with the nuclear membrane (fig. 7). Although the Swiss mice are generally thought to be free of the milk factor, these viruses are morphologically identical with the intracytoplasmic particles of the mouse mammary carcinoma. However, in the lymphoma cells, this type of intracytoplasmic particle is not associated with the extracellular particle consisting of four concentric membranes usually found in the mammary tumor.

The relationship of these particles to the leukemia virus and to the etiology of this lymphoma is still not known, since it has not as yet been possible to transmit the disease to adult mice with cell-free filtrates. Finally, it remains unclear whether this transplantable tumor was spontaneous in origin or related to the experimental procedures. It must be stressed, however, that among the lymphoma cells typical mature leukemia viruses are occasionally observed in intercellular spaces (fig. 9). Obviously this is a complex problem requiring further integrated biological and morphologic studies.

It is worth noting that particles similar to those observed in the lymphoma have also been observed in a mammary tumor of a DBA mouse by Dr. Dan Moore, and in a lymphoma in a Swiss mouse by Dr. A. J. Dalton and Dr. Alice Moore.

In conclusion, with the precise morphologic techniques in electron microscopy, it is now possible to differentiate the oncogenic viruses and to look forward to their classification on the basis of morphologic data. Within such a framework it would appear that the Swiss mouse leukemia virus differs from the following viruses: Rous sarcoma, chicken leukosis, Shope fibroma and papilloma, polyoma and mouse mammary carcinoma, as well as from the lymphoma particle. It resembles, however, the virus described by Adams and Prince (*11*) in the Ehrlich ascites tumor, and the agent described by Graffi (*12*) in a mouse myeloid leukemia. It is also similar to the Gross (*13*) and Moloney (*14*) leukemia agents.

In summary: The Swiss mouse leukemia virus was studied with the electron microscope. Lead hydroxide electron staining was found most helpful for the demonstration of virus fine structure. Special consideration was given to the successive steps of virus maturation by a process of budding at the level of the cell membrane and to the different localizations of the virus. Recent observations on particles of approximately 800 Å in size observed in a transplantable lymphoblastic lymphoma were also discussed.

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PLATES

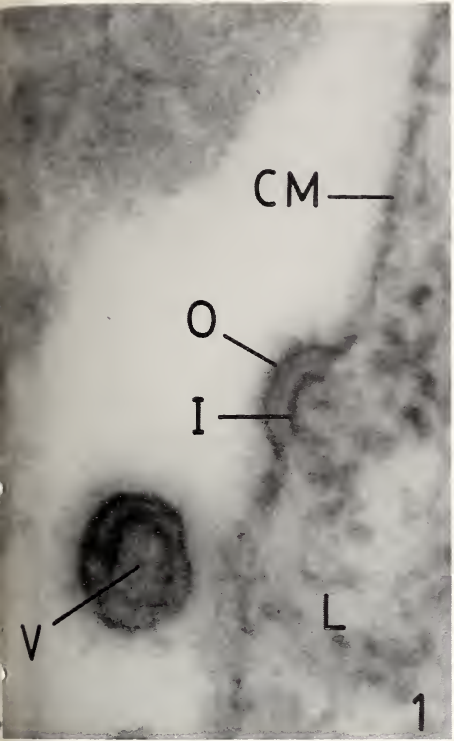
Plate 44

Figure 1.—Leukemic solid tumor. Lead hydroxide staining. At level of cell membrane (CM) of leukemic cell (L), virus bud is recognizable. Outer virus shell (O) and inner virus shell (I) can be seen at this early stage. One mature virus (V) is seen in intercellular space. × 217,000

Figure 2.—Liver of leukemic mouse. Lead hydroxide staining. Liver highly infiltrated with leukemic cells. A further step in differentiation of virus is seen. Particle is connected to cell membrane (CM) by large pedicle (P). × 180,000

Figure 3.—Spleen of leukemic mouse. Lead hydroxide staining. A completely formed virus is seen. Maximum electron density appears between outer (O) and inner (I) shells, the central part of virus showing only a moderate electron density. This virus is still attached to cell membrane (CM) of a leukemic cell (L) by an extremely thin pedicle (*arrow*). × 225,000

Figure 4.—Spleen of leukemic mouse. Lead hydroxide staining. Three viruses are seen in intercellular space. In two, the section has passed through central portion of particle and therefore their centers appear clear. Another virus is seen cut more tangentially. High density of inner virus shells (I) is observable, and in 1, intermediate virus membrane is seen between two virus shells (*arrow*). × 220,000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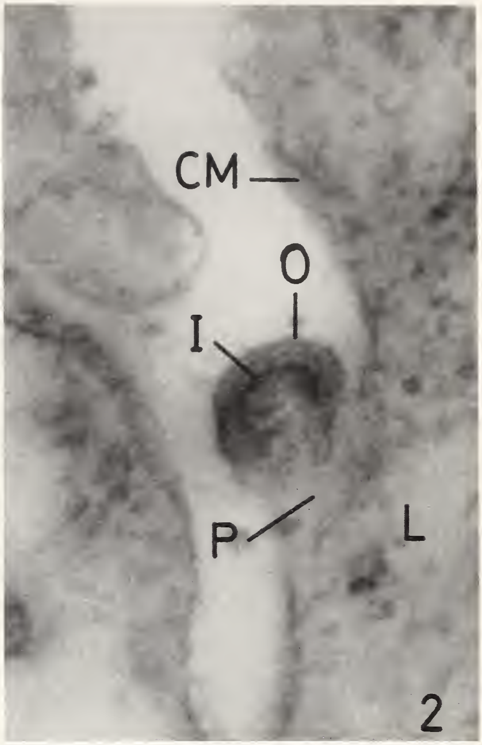
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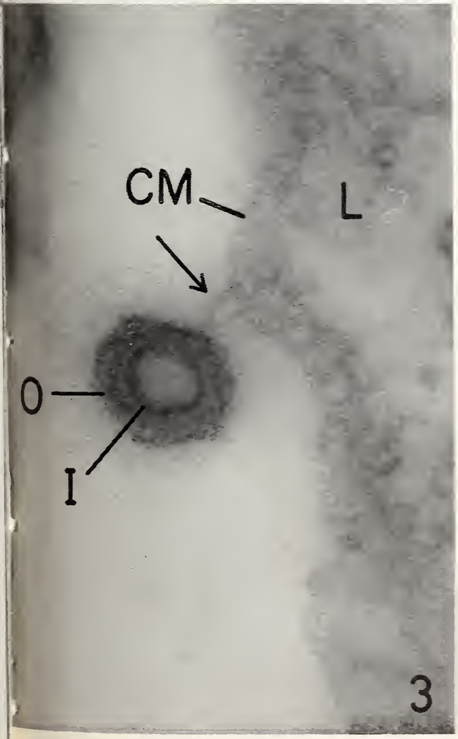
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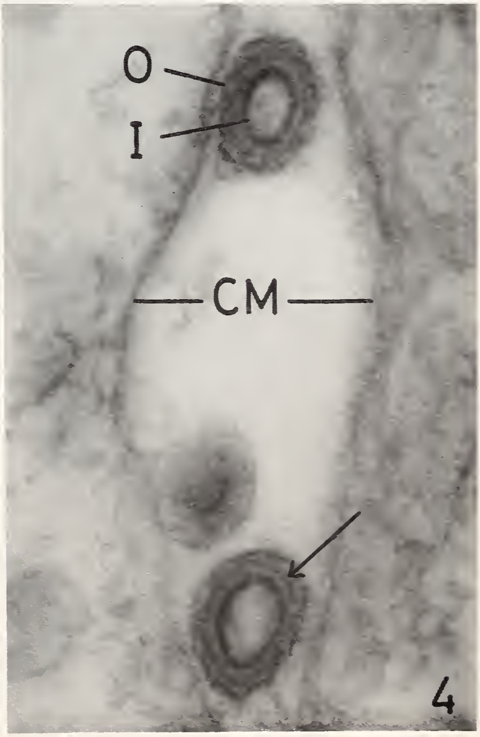
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4

PLATE 45

FIGURE 5.—Bone marrow of leukemic mouse. No lead hydroxide staining. In cytoplasm of megakaryocyte numerous vacuoles are seen containing many viruses. These viruses appear to be budding (*arrow*) from membrane limiting cytoplasmic vacuoles (M) by same process observed at level of cell membranes of leukemic cells. Structure of these viruses is identical to that of viruses observed in intercellular spaces of leukemic organs. The only particularity is the frequent occurrence of dumbbell (DP) or chainlet (CP) profiles resulting from the continuity between outer shells of several viruses. × 84,000

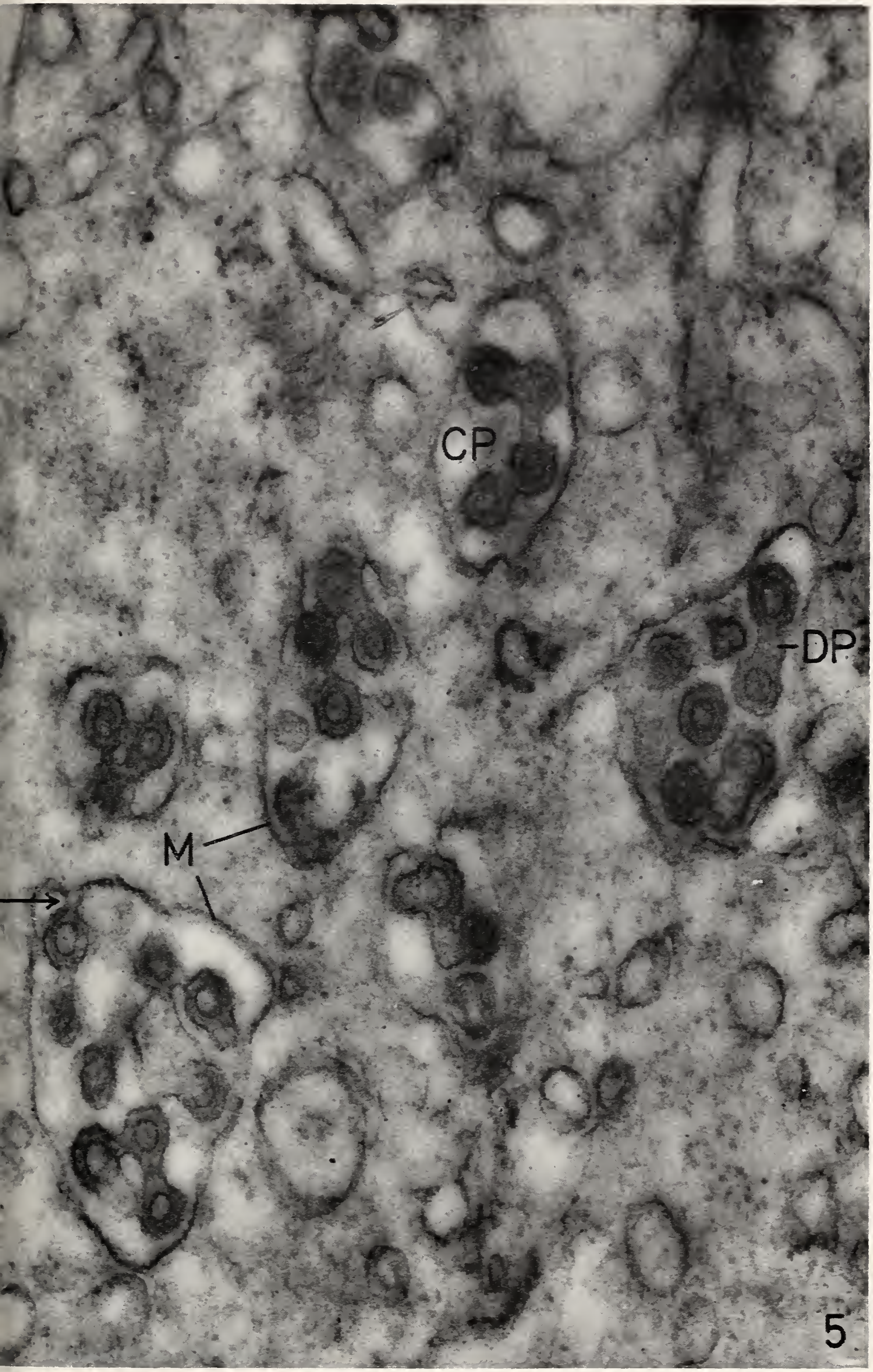
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Plate 46

Figure 6.—Involved thymus of mouse with lymphoblastic lymphoma. Uranyl nitrate staining. Mitotic figures are present (MI). In at least two cells an inclusion body formed by numerous particles is seen (*arrow*). × 5,500

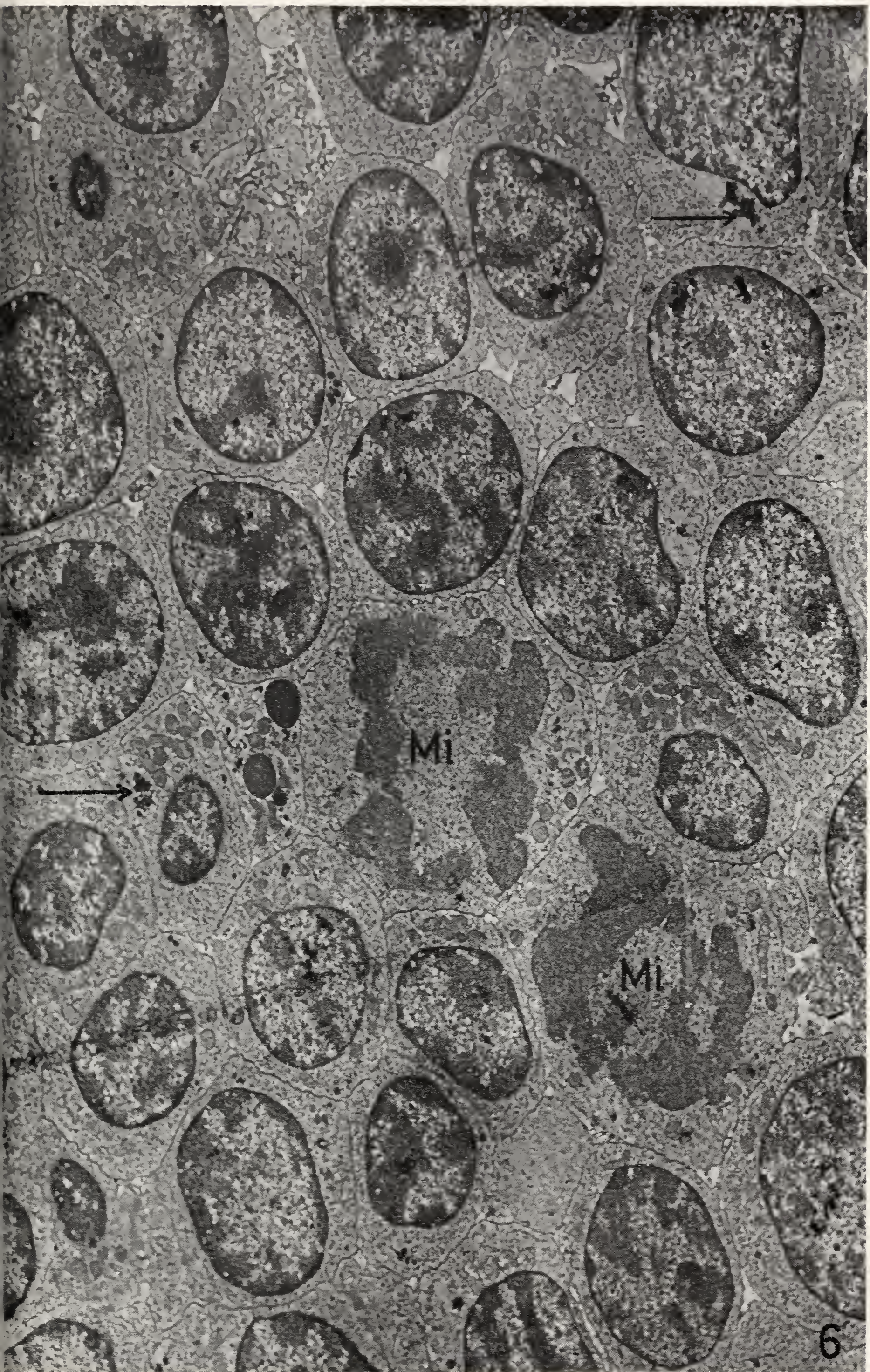
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Plate 47

Figure 7.—Lymphoblastic lymphoma from subcutaneous site of implantation. Lead hydroxide staining. Near nuclear membrane (NC), group of particles extremely regular in size is seen. These particles seem to have a close relationship to nuclear membrane (*arrow*). × 72,000

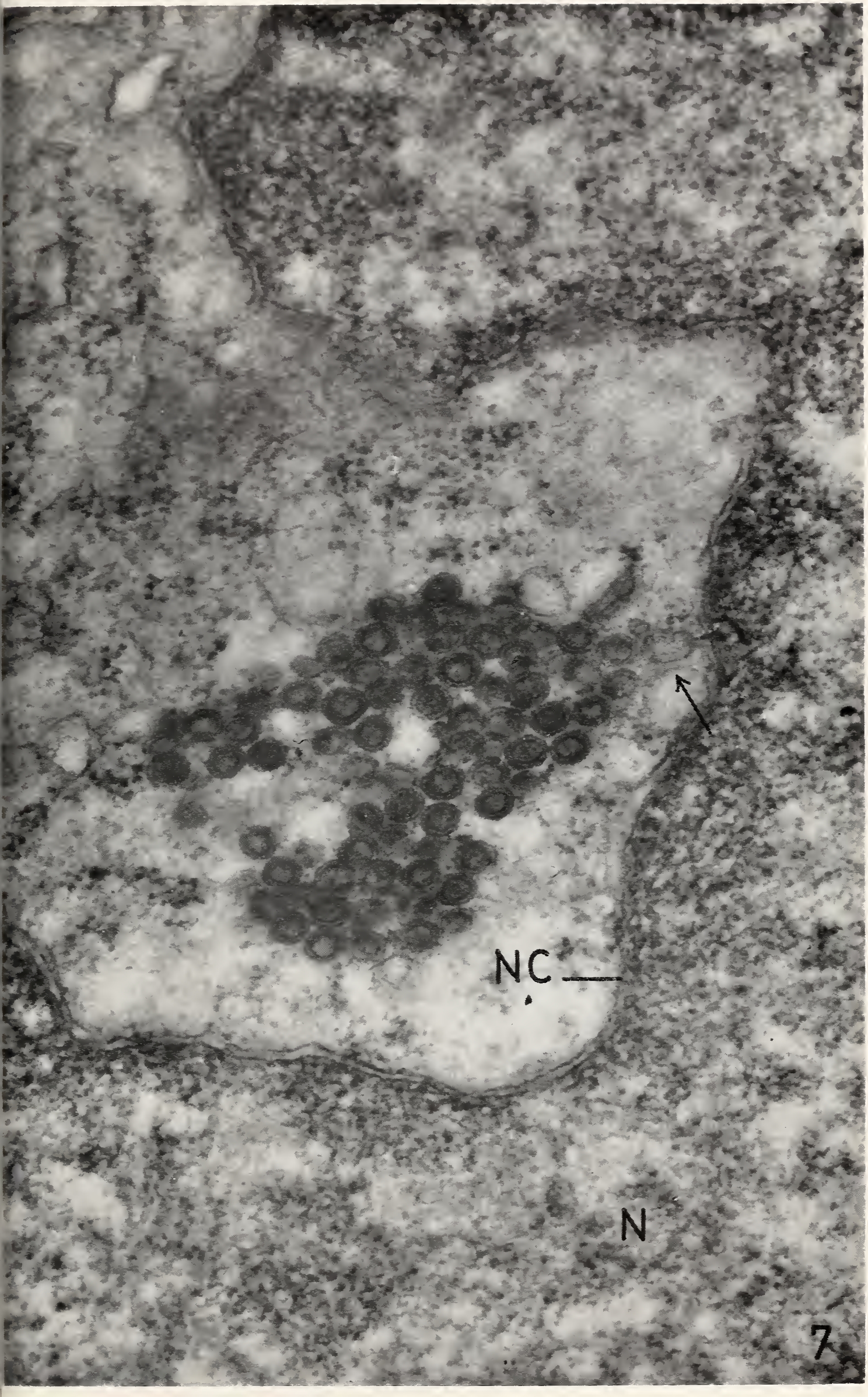
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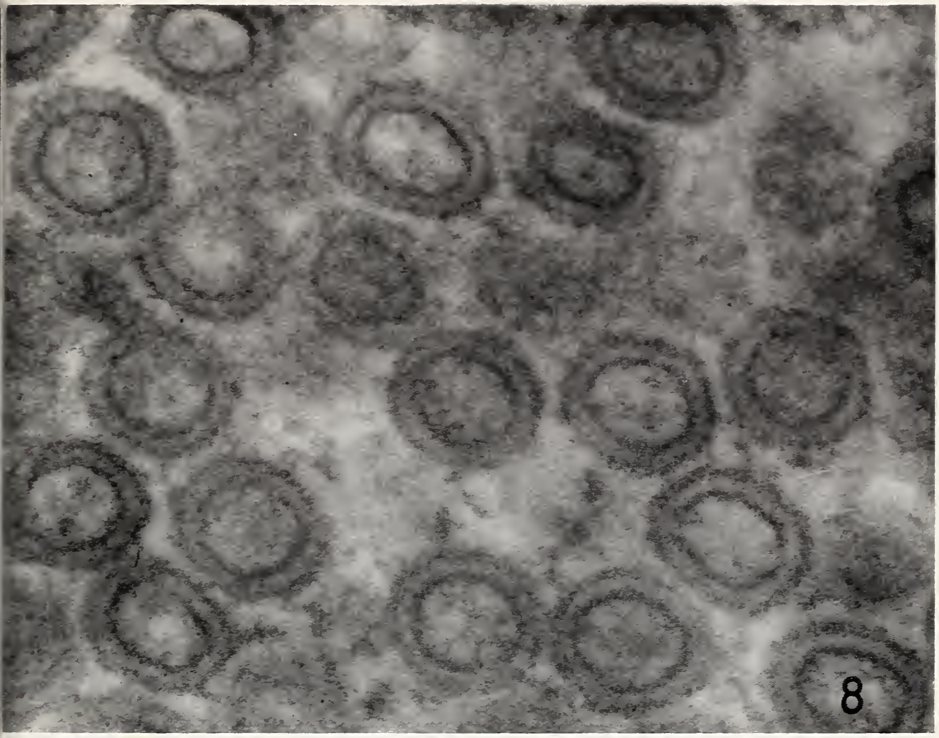
PLATE 48

FIGURE 8.—Lymphoblastic lymphoma from subcutaneous site of implantation. Lead hydroxide staining. High-magnification micrograph illustrating regularity of particles observed in lymphoblastic lymphoma and differences in shape between these intracytoplasmic lymphoma particles and the extracytoplasmic leukemia virus of Swiss mice. × 306,000

FIGURE 9.—Lymphoblastic lymphoma involving mesentery. Lead hydroxide staining. Between lymphoblastic cells of this tumor, rare but typical leukemia viruses are seen in intercellular spaces (*arrow*). × 145,000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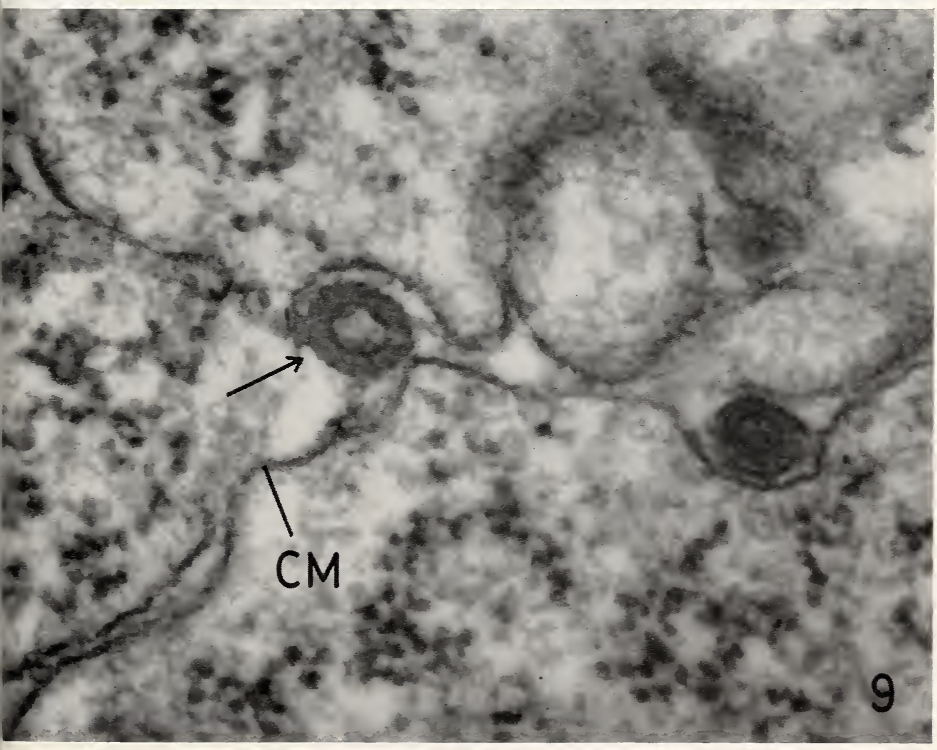
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Discussion

Dr. Fogh (New York State Health Department): It has been striking to me that in this otherwise wonderful seminar the information concerning work with human cells has been very limited. I would like to discuss recent results from my work with human amnion cells in tissue culture.

We found 4 years ago that cultures of amnion cells, like cultures of many other normal cells, could change to a type of cell which, in many respects, had the characteristics of malignancy. Since then we have been looking for some means of measuring quantitatively the "transforming" capacity of a culture, and we have recently had some quite encouraging results.

It was first observed that in cultures, transferred 3 weeks after the original seeding, cell colonies could develop in the transfer culture. At first the colonies consisting of changed cells were visible only when the cultures were observed in the microscope. As they grew out, they could be seen macroscopically and could be counted easily. The cultures, each originating from a seed of two million cells, contained an average of approximately 200 colonies of changed cells. There was a clear distinction between the colonies of changed cells and the degenerating primary cells which could be observed in the spaces between the colonies.

The same type of phenomenon was observed in primary cultures. Visible, distinct colonies of changed cells, easy to count, have been observed in 2- to 3-month-old cultures. It has been interesting to note that in several cases in which the distribution of the primary cells was uneven, the major part, in some instances practically all, of the colonies of changed cells occurred in the parts of the culture in which the density of unchanged cells was lowest. This observation agrees with Rubin's observation of the Rous virus transformation system in tissue culture, and it could be explained likewise by an inhibiting effect of the unchanged cells upon the development of the changed cells.

In another type of experiment we have studied the effects of filtrates prepared from changed cells (FL cells). The cells were broken down and the cell material was passed through a series of Millipore filters. Primary amnion cultures inoculated with filtrates showed changes not observed in uninoculated cultures. Filtrates passed through the 450 or 300 mμ Millipore filter have, within 2 weeks after inoculation, caused macroscopically discernible plaque-type lesions in the cell sheets. The lesions were not exactly countable, but were similar in gross appearance to various plaque types we have seen in monolayers infected with animal viruses. The plaquelike lesions, however, were different from the type seen, for instance, in polio virus-infected cell sheets. When observed in the microscope, they were filled with rapidly growing, small, clear cells with a morphology different from that of cells outside the plaquelike areas.

We have one more experiment in which the effects of filtrates have been noted. Twelve amnion cultures were inoculated with Millipore filtrate, and 12 cultures were left uninoculated. Several months later changed cells appeared in 5 inoculated cultures, whereas nothing happened in any uninoculated culture.

I am not prepared to interpret these findings. There are still many control experiments which have to be carried out. I thought, however, that this observation was worth presenting at this seminar to show that interesting things can also happen to *human* cells.

In spite of great progress in virology, many points are still confusing, but once in a while the virus particles help us by getting into a controlled, orderly system—for example, when particles of polio virus arrange themselves in crystalline arrays in the cytoplasm of infected HeLa cells.

Dr. Dalton (National Institutes of Health): I want to acknowledge the beautiful demonstration of material shown by Dr. de Harven. I can agree with his remarks, in regard to the Moloney agent and its great similarity to the Friend virus, that in the beginning the particles are the A type and then apparently, on aging, become the C type. However, the size of the particle itself is considerably greater, closer to 100 mμ than 85 mμ.

In the cell of origin of the Moloney agent, presumably in Sarcoma 37 in which the particles are present intracellularly, the particles have a single outer membrane of 86 mμ diameter, while those in an extracellular position have a diameter closer to 100 mμ.

It should also be mentioned that Miss Mottram and I have been examining the material provided by Dr. Alice Moore, and in several lymphomas derived from different sources there were seen doughnut-shaped particles similar to those described in Dr. Friend's material.

Dr. Bonar: Will Dr. de Harven elaborate on the difference between the extracellular A type and the intracellular doughnut-shaped particles?

Dr. de Harven: The difference is especially obvious after lead hydroxide staining. After such treatment, the inner shell of the leukemia virus appears extremely dense, far more so than the outer shell. In the lymphoma particle there is not the same difference, and the ratio between the diameters of the outer and the inner viral membranes is also different.

Dr. Gey (Johns Hopkins Hospital): From the results presented it would appear that the virus particles in many cases are increasing in number. The question is: How do they increase as morphologic elements? In your situation, it would appear that a virus makes contact with the plasma-cell membrane and a new virus is grown. Is that right?

Dr. Beard: Dr. Gey wants to know what you think about the mechanism of replication, how the virus increases, and what is occurring at the membrane.

Dr. de Harven: No information is available concerning the lymphoma virus particles but for the leukemia particles it would appear that what is seen is probably an ultimate step in the duplication of the virus material.

Dr. Gey: Let us consider, then, another matter. In all the studies on viruses in terms of morphologic display, is there any evidence of simple binary fission? Is it possible even to speculate on how the virus grows as individual elements—in the cell or out of the cell or anywhere else?

Dr. de Harven: We have no evidence of binary fission at all. That is a proviral stage which has thus far evaded our morphologic techniques.

Dr. S. A. Rose (University of Illinois): In the last 2 days we have heard about some marvelous techniques for the study of the interaction of viruses in cells, viruses in tissues, and viruses in the whole animal, including immunologic methods and the examination of the tissues by microscopy. What have all these to do with cancer? I ask this provocative question for this reason: Very few cells during the process of carcinogenesis actually become malignant. How do you examine the process in some cells, when they are surrounded by other cells in which that particular and specific process is not actually occurring?

Dr. de Harven: You mean the exact distinction between a cancer cell and other cells?

Dr. Rose: In examination of the cells by any technique described, how do you know you are examining the specific process by which a cell is likely becoming malignant? You are examining the interaction between virus and cells, but you are not examining the particular process by which the odd cell becomes malignant. Consequently, the last 2 days have been a fine seminar on virology; I cannot say in any way that it has been a seminar on the study of carcinogenesis.

Dr. Beard: I do not think you should go quite that far.

Dr. Rose: Let me put it this way. When one feeds an animal azo dyes, one gets certain changes in the liver. One of those changes is a loss of glycogen from the liver cells. Not many people would say that the loss of glycogen, which occurs in every liver cell, has something do to with this specific process by which the one cell of many becomes malignant; this is the problem you face with your virus interaction with cells and tissues.

Dr. Beard: No, this is not the problem under consideration here. At the time we undertake to talk about tumor viruses and their relationship to the cell, we have already passed the point at which we know about the malignant state of the cell under consideration. For instance, in myeloblastosis, there is no doubt that the cell being studied is malignant. Actually, the nature of the biochemical processes that cause the cell to become malignant is an entirely different problem remaining for study.

Dr. Rose: No, the question is really that, by the time you see the malignant cell and the virus particles in it, it may then be that the malignant cell has become infected with some nonspecific virus. This has nothing to do really with the question of whether the virus under study actually induced the carcinogenic process in that particular cell.

This is not the problem of carcinogenesis at the moment. It is a problem of microheterogeneity and response, in that it occurs only in a few cells and nothing is to be gained by examination of the whole tissue. In fact, the carcinogenic process could be developing in one series of cells which are not actually infected with the virus and you would not know about it.

Dr. Beard: Your remarks are not particularly relevant to the matter of this meeting. You certainly have no basis for questioning the knowledge of the malignant state of the cells under study. The myeloblasts about which we have spoken are certainly neoplastic, and without virus malignancy does not occur. Within the limits of the evidence thus far obtained, these cells—every one of them—contain virus. There is no reason to suppose that virus is absent from any Rous sarcoma cell, and the same thing may be said for mouse mammary carcinoma.

Dr. Rose: The process of carcinogenesis may not have occurred, however, in the cell that contained the virus. It may have occurred in some other cell. When you see the process at the end and you see a whole malignancy, then the cells are infected with the virus. That, however, does not mean to say that the specific and rather rare sort of process in which one or two cells become malignant can be examined by examining the whole tissue. That should be fairly clear. If a process is occurring in one cell in a million, how do you detect that process by examining the whole million cells together?

Dr. Friend: May I suggest one thing? Dr. Rose has raised a provocative point and, in so doing has devised for himself a wonderful problem to carry home with him. He can take a thousand cells, isolate them one by one, study what they produce, do exactly the same thing with another thousand normal cells, putting one little virus into each little cell, and come back in 10 years to tell us what he finds.

Dr. Dmochowski (M. D. Anderson Hospital): I think this was a most unfair question. The symposium was meant to be concerned with phenomena of tumor viruses and not with the means by which viruses induce cancer. This is quite a different subject about which so little is known. But I would ask Dr. Rose how much he knows about the process of carcinogenesis. Induced by anything? By benzanthracene? Any dye stuffs? He knows as much (I hope—perhaps I am wrong, perhaps he does) as we do about the way viruses induce cancer.

Dr. Sabin (Childrens Hospital Research Foundation, Cincinnati): I suppose I have as little or as much right as anyone else to comment on this broad question, and I would like to say what I am carrying away with me after these 2 days.

We came here, it seems to me, to analyze the processes in a number of tumors, with which viruses have been associated, in a way that one can reproduce those tumors with the respective viruses. The association of the two is at least established, and now we have been trying to solve, in effect, the very question that Dr. Rose raised. How are these viruses involved with the formation of the tumors?

I am interested in that problem, particularly to find some rational approach to what may be done in the study of human tumors, when it is not possible to utilize 1-day-old humans for inoculation with an extract and then wait several years to see if they will develop tumors like those produced by the polyoma virus. We are searching for guides in all these processes to see how so-called tumor viruses may be associated with the end product. I gather that, for one thing, not all tumor viruses are working with their tumors in the same way. Let us take these avian leukemias that Dr. Beard and his associates have been working with so long. He gets so much virus there that I should think he ought to be able to get it in large quantities from the blood. It seems that the whole gist of the discussion was whether a malignant cell that continues to multiply is producing the virus, or is it perhaps that the virus comes from the dead cell? I have been trying to decide from the work his associates have been presenting whether it is the 5 percent of the cells which die that produce all this virus or the 95 percent that remain alive.

It is not impossible for me to believe that the cell can continue to divide and produce some virus, so it may well be that some malignant cells are multiplying and yielding virus, but others not. I think the most important matter now coming into focus is the polyoma model. In this system, Dr. Rose, the virus is multiplying in millions and billions of cells, from one or a few of which, after many months, there develops a tumor. It is most striking and important that the tumors, after several transplantations in virus-free hamsters, do not yield any virus or any evidence of viral antigen or viral reproductive material. And now, the question arises: Is this the polyoma virus-induced tumor cell in which we cannot demonstrate any trace of the original virus, such as its nucleic acid or its provirus? Or, does the tumor now consist of cells that bear the same relationship to the polyoma virus as the cells of a malignancy induced by methylcholanthrene bear to the chemical? This cannot yet be answered. There are being studied different modes of action of viruses which, with regularity, result in the induction of tumors. Many mechanisms may be involved, and that all the questions have not been answered is not disturbing at all, since, with the reports at every new meeting, a little more light and a little more progress are introduced.

Dr. Beard: The time has come to close this symposium. I should like, personally, to thank all of you for coming, and I know that the members of the Virology and Rickettsiology Study Section are gratified to know your interest in the conference.

It was the aim of this program to bring together people of widely different disciplines in the attempt to arrive at some general understanding of the total problems. It is unlikely, of course, that a close correlation can be effected of the findings in this presently complex and confused field. It might be possible, however, to bring the numerous people now working with virus tumors somewhat closer together.

There has been much effort in recent years, by what might be called summit meetings on the tumor-virus problem, to find a short cut to the solution of the numerous questions in this field by a rather indiscriminate exchange of ideas by workers in widely separated areas. While it cannot be denied that such exchanges may be highly stimulative and entertaining, it is certain that the problem cannot be talked to a solution. This can be attained only by the careful and progressive accumulation of information by investigators like you who are intimately concerned with direct experimentation.

Comparative Micromorphology of Normal, Transforming, and Malignant Cells[1,2]

GEORGE A. EDWARDS, *Division of Laboratories and Research, New York State Department of Health, Albany, New York*

THE purpose of micromorphologic comparisons of normal and malignant cells has changed rapidly within the past several years, due to improvements in microscopy, increased identification of morphologically recognizable cellular components, and increased availability of controlled experimental material. It is no longer sufficient, at the micromorphologic level, to describe merely size and shape of nuclear and cytoplasmic organelles. It now becomes our obligation to investigate structures of molecular dimensions with the particular view of determining (*a*) sites and modes of exchange of genetic information within and between cells, and (*b*) the relation of this exchange to normal and abnormal synthetic processes. This is particularly necessary in the study of virus-tumor cells in view of the close relationship, or exchange, of viral and cellular genetic information.

Detailed structural studies, in normal cells, of such processes as spermatogenesis, nucleo-cytoplasmic exchange, and nucleolar-chromosomal interrelations have already suggested the possibility of gaining an insight into normal genetic control of synthesis. Further, the visualization of cell border activity, ribonucleoprotein synthesis, and endomembrane continuities has opened the door for the visualization of pathways of exchange between environment and controlled cellular systems. Numerous investigators have afforded us a steadily increasing body of information on the ultrastructure of tumor cells, virus-infected cells, and viruses themselves. More pertinent to the study to be presented in the following pages is the recent work on the fine internal structure of virus particles and tumor cell inclusions. Specifically pertinent is the visualization of the coiled thread structure of poliovirus (*1*), polyoma (*2–5*), adenovirus (*6*), and pox virus (*7*). Related to this is the recent demonstration of inclusion-body fine structure in myeloblastosis (*8*, *9*), stomatitis (*10*), swine pox (*11*), and parotid tumor cells (*3*).

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[1] Dr. Edwards was invited to present this paper at the Symposium on Phenomena of the Tumor Viruses, New York, N.Y., March 25 and 26, 1960. Because of his death March 1, 1960, the paper was not given.

[2] This investigation was aided by grant P 145B from the American Cancer Society, Inc., and by grant C-4926 from the National Cancer Institute, National Institutes of Health, Public Health Service.

The survey to be presented is less a comparison of normal and malignant cells, but rather, more a comparison of virus-infected cultured cells, transforming cultured cells, and virus-induced tumor cells. It is hoped to find certain common denominators that can serve as clues to the determination of sites and modes of virus formation, and of the relation of these to normal and abnormal cell processes.

NUCLEUS

The nucleus normally comprises peripherally arranged chromatin, one or more nucleoli and nucleolar fragments, and a nucleoplasm containing various granules and fine filaments. The nucleus is bounded by an envelope continuous with membranes of the endoplasmic reticulum, and facultatively continuous, thus, with the plasma membrane of the cell. At various intervals the nuclear envelope is interrupted by the nuclear pores, affording continuity of nucleoplasm and cytoplasm. In normal cells the nuclear organelles pass through morphologic cycles in association with growth and metabolic events. Some nuclear changes observed in transforming and tumor cells are similarly related, *i.e.*, increase in nuclear size, nucleolar size, vacuolization and fragmentation, and increase in numbers and width of nuclear pores with the increased synthetic activity of the cell (*12, 13*).

Viral particles, or viral precursory material, have been observed in the nuclei of numerous infected cells, *e.g.*, herpes (*14, 15*), polyoma (*2–5*), Friend leukemia agent (*16, 17*), and adenoviruses (*18, 19*). The question immediately arises as to how such material is transmitted to the cytoplasm. The mode of transmission apparently varies with virus type. Herpes virus, for example, may pick up a membrane from the nuclear envelope and probably be transmitted across the envelope by reverse pinocytosis. Cytopathic viruses, *e.g.*, poliovirus and hemadsorption virus (*20*), may cause breakdown of the nuclear envelope or enlargement of the nuclear pore to the point where the particles may simply diffuse directly into the cytoplasm. Noncytopathic viruses probably use the normal pathway of nucleo-cytoplasmic exchange, *i.e.*, the channel of the nuclear pore. Evidence has been accumulating for the normal nuclear control of cytoplasmic processes by the production and migration of ribonucleic acid (RNA) within the nucleolus and the subsequent passage of nucleolar material through the channel to the cytoplasm. It is interesting to note that in the case of poliovirus (*21*), nucleolar material seems to pass to the cytoplasm, re-form a well-defined nucleolus, and that the filaments of the cytoplasmic nucleolar structure appear to be in direct association with material involved in poliovirus crystal formation. In the parotid tumor cells of polyoma-infected mice the intranuclear polyoma particles (figs. 1, 2, 3, and 4) appear to be closely associated with nucleolar filaments in the formative stages (fig. 1). In the leukemic thymus we have observed filamentous material in the nucleoplasm near the pores, within the pore channels, and continuous filaments from pore channel to cell border, where definitive virus particles are forming (fig. 12). The nucleolar material could thus be directly involved in virus formation. However, as in normal synthetic processes in the cell, the nucleolar material probably provides the transport mechanism and it is nucleolar-associated material that passes through the pore from nucleus to cytoplasm. Further, it is apparent that, in some virus tumor cells, particles *per se* do not pass from nucleus to cytoplasm, but rather a viral material in the form of fine filaments passes through the nuclear pore channel.

Cytoplasm

The endoplasmic reticulum, as might be expected from its importance in nutrition, conduction, and membrane synthesis within the cell, does not seem to play a direct role in tumor virus replication transmission and extrusion. The variations in endoplasmic reticulum observed in infected, transforming, and tumor cells seem to be more related to metabolic variations and membrane synthesis than to virus production. In transforming human amnion cells, the endoplasmic reticulum changes from smooth-profiled to rough-profiled vesicles and tubules during growth of the cell (*12*). In FL cells in horse serum, as also in certain tumor cells, fenestrated lamellae of the reticulum have been observed (*13*). High magnification shows the dense regions of this structure to be intermembrane "desmosomes," hence normal components. In nonviral tumors, *e.g.*, bronchogenic carcinoma (*22, 23*) and squamous-cell carcinoma (*24*), the endoplasmic reticulum may enter into the formation of cytoplasmic filaments or myelinoid forms. However, such forms occur in some normal cells and hence cannot be directly related to cell abnormality. Perhaps the closest relation of the endoplasmic reticulum to a viral form is that seen in poliovirus-infected cells, in which the polio particles in the cytoplasm appear to be laid down in patterns determined by the ergastoplasmic membranes (*21*).

The involvement of the Golgi complex in infected and in tumor cells is consistent with its normal function in any cell. The Golgi complex comprises dictyosomal lamellae, macro- and microvesicles, and microvesicular bodies. The dictyosome and macrovesicles appear to play a role in normal feed-back mechanisms within the cell. The microvesicles and microvesicular bodies apparently are more concerned with secretion and elimination. Microvesicles are most commonly seen in those cells which normally are secretory, *e.g.*, pituitary, pancreas, hypodermal gland cells, and the like. Microvesicular bodies most commonly appear during hyperactive stages of cell growth or metabolism, *e.g.*, during the early stages of tissue-culture growth, during chloride secretion of gastric mucosa cells, and during virus release from infected tissue-culture cells. In its function as an excretory organelle, then, it is not surprising that the Golgi complex should contribute to the release of viruses from the cell, and perhaps actively contribute membrane material, as in the formation of herpes (*14*, *15*) and mammary-tumor viruses (*25*).

The principal cytoplasmic organelle involved in parotid tumor cells and leukemic thymus, as in myeloblastosis and swine pox-infected cells, is the mitochondrion. In normal and transforming cells, as in most virus-infected tissue-culture cells, the number, size, and shape of mitochondria appear to vary directly with metabolic stage. In the polyoma- and leukemia-infected cells the mitochondria also play a role in the formation of inclusion bodies (*3*, *26*). I would like at this point to redefine "inclusion body" on the basis of the evidence to follow. Inclusion bodies as defined by light microscopy are identified on the basis of their size and stainability. In electron microscopy they have been variously defined but probably most often given as virus-containing vacuoles of the endoplasmic reticulum or Golgi complex, or as virus-containing, dense structures originating from mitochondria. The present evidence supports the latter view, and suggests that not only virus-containing structures, but also the previously described "gray bodies," "dense bodies," and other similar structures found in virus-tumor cells should be included within the category of inclusion bodies. In osmium, and possibly chromium, stained leukemic thymus, and polyomatous parotid cells, it is possible to identify not only the definitive virus-containing inclusion body, but also the transforming mitochondrion and early stage inclusion body, even though no particles are visible within the matrix.

The apparent sequence of events in the formation of the inclusion body in the parotid tumor of the polyoma-infected mouse is as follows: The normal mitochondria are small, roughly cylindrical, possess few cristae, and little matrix (fig. 8). Some show mitochondrial granules; others are smaller, possess fewer cristae, and appear to be more spherical in outline. Modified, but still clearly recognizable as mitochondria, are structures with dense double outer membranes, vestiges of cristae, but with densely filamentous matrix. Large structures in such an area appear to be formed by the aggregation of these latter forms. In some of these large forms there may still be seen vestiges of outer mitochondrial membranes and remnants of cristae. An apparent later stage is represented by similar structures showing no membranes within, but only a finely granular to filamentous dense matrix. Within such forms dense spots are sometimes observable, usually at the periphery (fig. 8). In others, identifiable single virus particles, or even crystalline arrays of virus particles, may appear (fig. 10).

In the leukemic mouse thymus the inclusion bodies are apparently also derived from modified mitochondria, albeit either in two different manners or representative of two types of body. One type of formation is similar to that seen in polyoma-infected cells. The second type shows characteristic fractionation of the mitochondrial cristae with subsequent symmetrical arrangement of the subunits, and a secondary transformation of these into filaments possessing an extremely fine axial period (fig. 9). In all definitive inclusion bodies there have been observed orderly arrays of dense, fine, periodic filaments, and aggregates of tiny dense spots apparently representative of viral particles or precusory viral material. The structure and density of the filaments are comparable to those of the filaments seen within the nucleoid of the mature leukemia virus particle.

The significance of the viral material, or crystalline arrays of particles within the cytoplasmic inclusion body, is still uncertain. It is even possible that the crystals are of nonviral protein. However, on the evidence given, it is most logical that the formation of viral particles and their crystallization in the inclusion body are merely due to the assumption of minimum form under conditions of high concentration or supersaturation. The transmission of the virus from the inclusion body to the cell border and its subsequent release become the next questions. It is doubtful that, in the Gross leukemia agent and polyoma, the particles observed in the inclusion bodies actually migrate as such throughout the cytoplasm. It is more suggestive that the elementary units of the component filaments are the migrating material, that these units recombine in whatever locale be best suited, and that the form so assumed would be the minimum possible for aggregated twisted filaments.

MATURATION AND RELEASE OF THE TUMOR CELL VIRUS

If these interpretations of our results be true, then the question arises not only where and how the viral substance migrates, but also where and how the mature virus is formed and released. Information is available from our studies of two of the tumor viruses (*3*, *16*) and one virus in tissue culture (*20*). In the following discussion I will illustrate only the formation of the Gross leukemia agent in the mouse thymus.

Intranuclear forms of the virus, similar to the nucleoidal form of polyoma (*2–5*), or the membrane-bound form of herpes (*14*, *15*), have not been observed in the leukemic thymus. In general, the nuclei show only the usual distribution of nuclear granules and chromatin. However, in two instances, particles, or granules, distinctly different from the rest of the nucleoplasm have been seen in nuclear areas of little density. These particles are of considerable density and are usually held together by a homogeneous material in large aggregates or clumps. Further, the individual particles near the edge of a clump appear to be more closely associated with nucleolar material than with other nuclear elements (figs. 5, 6, and 7).

Definitive cytoplasmic forms of the leukemia virus have not been observed. As noted, inclusion bodies (fig. 9) containing ordered arrays of fine filaments or dense spots have been seen. Further, threads of material similar to the modified mitochondrial cristae are seen scattered throughout the cytoplasm (fig. 9), but more often close to the cell border. In all preparations of the leukemic thymus, ordered aggregates of fine filaments, which possess an axial period and side brush-like filaments, are observed at the cell border, usually in association with developing particles (figs. 11 through 13).

The definitive leukemia virus forms only at the cell border. Its formation appears to involve both the peripheral filamentous material in the cytoplasm and the modified plasma membrane (figs. 13 through 16). The components of the plasma membrane form twisted filaments also at the point of virus formation. These filaments have a period and structure similar to the peripheral cytoplasmic filaments (figs. 13, 15, and 16). Thus the initial step in the virus formation is a combination of filaments of the cytoplasm and the cell border, which appear as a dense thickening and protrusion of the cell surface. Increase in size of the virus is brought about by increased aggregation of the filaments into larger, spiralling threads. These apparently first form the outer limits of the virus, and then, by close packing, form an ordered array within the central portion of the particle (fig. 17). Among the threads occurs a matrix material, similar to that in the cytoplasm, surrounding the peripheral filaments. While still attached to the cell surface the virus shows reorganization of material such that a distinct nucleoidal area is apparent, surrounded by incomplete threads giving the appearance of an outer coat three to four membranes thick (figs. 13 through 20). As the virus particle leaves the cell border, there is an apparent condensation and fusion of the filamentous material to give the final form of nucleoid, the various components being separated by a matrix material of little density (fig. 21).

Sections have been cut in planes parallel with and within the cell surface, as well as secantial and normal to the surface. Thus it has been possible to follow the sequence of events in the particle formation from different views. By this method it has also been determined that the cell surface is highly irregular (fig. 14). From the determination of the cell contour and the method of virus formation it is concluded that the definitive virus forms only at the cell border, and that previously described virus-containing vacuoles in the cytoplasm are probably depressions in the virus-forming cell surface.

SUMMARY AND CONCLUSIONS

The significant points to be brought out in the present discussion are as follows:

1. In direct correlation with biologic evidence, the electron microscopic evidence shows that tumor virus particle formation, in precursory or elementary form, occurs in association with genic material in the nucleus, and with enzymatic sites in the mitochondria, resulting in modification of these organelles.

2. At least in several of the viruses studied in the present investigation, the elementary viral substance is similar in size and structure to the elementary substance of the normal genetic coded material, that is, the viral unit substance is composed of twisted filaments of the dimension of the alpha helix.

3. In the formation of the tumor virus, at least in the Gross leukemia agent, the elementary filamentous units enter into the formation of the

coat as well as nucleoid of the virus. Hence the viral coded information exists in all parts of the mature virus.

4. It can now be explained why investigations to date have not shown entering or migrating virus particles, and perhaps why virus particles as such have not been visualized in human tumor cells.

5. As shown in several instances mentioned, and in one instance of filamentous material continuous from nucleoplasm through the pore channel and cytoplasm up to the point of virus particle formation at the surface, it is in the twisted filamentous form that the leukemia, and possibly polyoma, virus material exists in the nuclear and cytoplasmic matrices. Replication would appear to consist of formation of an excess of filamentous units. These in turn form aggregates or crystals within the cell at optimal sites under the usual laws of crystal formation and concentration phenomena. The mature virus is apparently formed only at the cell surface prior to release.

6. The question arises as to whether the modifications of the affected cell are brought about by changes in its own coded information due to propinquity of viral information, or whether the viral information controls the pathways and processes of the cell, bringing about local modifications directly related to its formation and release. The latter seems more logical. The entry and initial migration could be as for any substance, be it neutral or active, *i.e.*, by pinocytosis and/or membrane flow. The replication and release apparently are brought about by changes in normal pathways due to the new information contained in the viral material. The cytopathic or noncytopathic effect of the virus would thus also depend upon the closeness of fit of viral and cellular information codes.

7. Now that it is possible to visualize the structure of the viral components at the molecular level, it becomes imperative to devise methods for distinguishing between normal and viral units in nucleus and cytoplasm, as well as within the mature virus itself. Included in the information to be thus derived would be that on latency, mutation, and contamination. A hopeful step in this direction has been provided by the chromium stain developed by Bullivant and Hotchin (*27*). In preliminary experiments there is some indication that it is possible to develop a method that specifically stains the viral material only.

Whether this particular method is successful or not, the results obtained so far indicate that the next step is not merely to look for or recognize large viral particles, but to distinguish viral from normal subunits at the level of the most elementary structures of the cellular organelles.

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PLATES

Plate 49

All figures are of mice tumor cells. Those infected with polyoma virus are from a parotid tumor and those with leukemia are from a thymus lymphoma.

Figure 1.—Nucleus (nu) of polyoma tumor cell showing relation of nucleolus (ncl) to fine filaments (f), with which virus particles (v) seem to be associated. Adjoining cytoplasm (cy) and nuclear membrane (nm) are seen in *upper* part. × 60,000

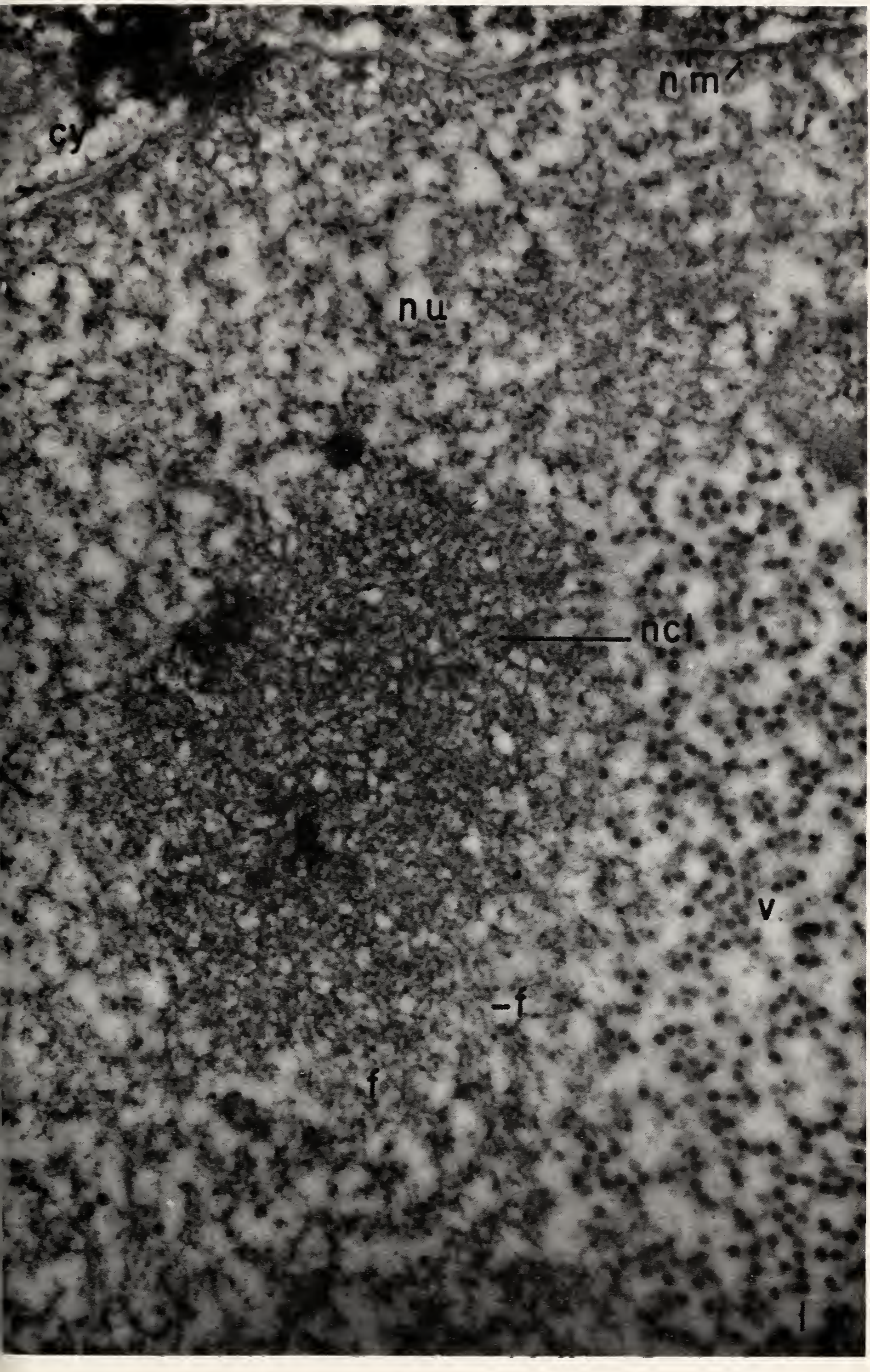
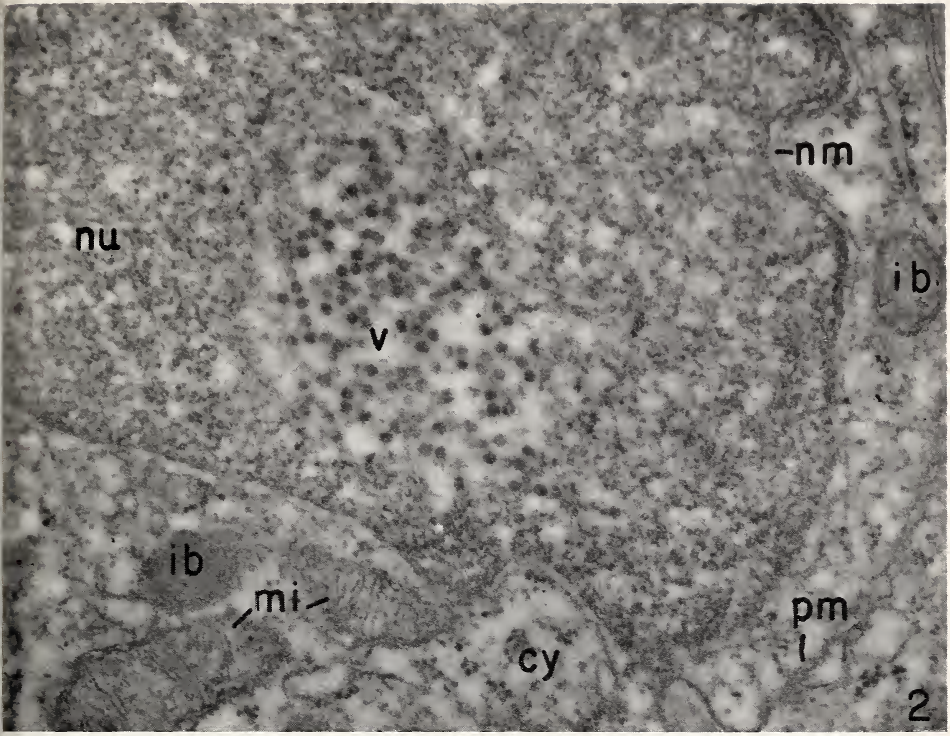

cy
nm
nu
ncl
v
f
f

Plate 50

Figure 2.—Nucleus (nu) with patch of viruses (v) in polyoma tumor cell. Nuclear membrane (nm), part of the plasma membrane (pm), and cytoplasm (cy) containing mitochondria (mi) and "inclusion bodies" (ib), as described in text, are seen. × 60,000

Figure 3.—Portion of cell of polyoma tumor showing viruses (v) in nucleus (nu) and nucleolar filaments (*upper left*). Cytoplasm (cy) is limited by plasma membrane (pm) and contains mitochondrion (mi). × 60,000

Figure 4.—Highly magnified intranuclear particles as seen in figures 2 and 3. *Arrows* point to fine twisted filaments of the internal virus structure. These seem to represent sections of the total ball-of-yarn-structure of virus nucleoid. × 300,000


nu
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-nm
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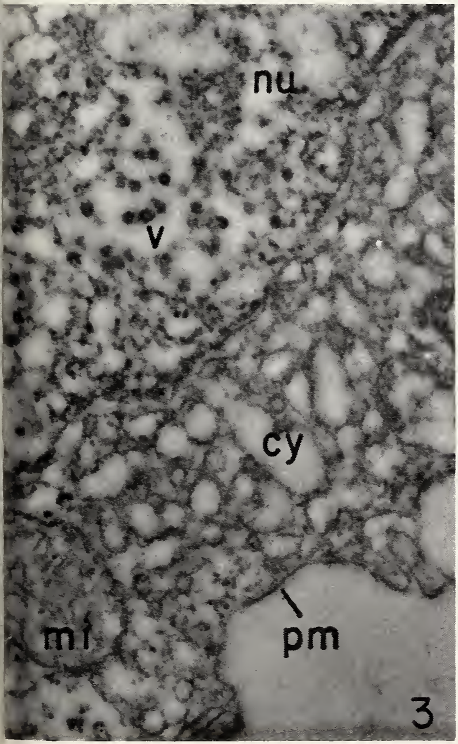
ib
mi
cy
pm
2


nu
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cy
mi
pm
3

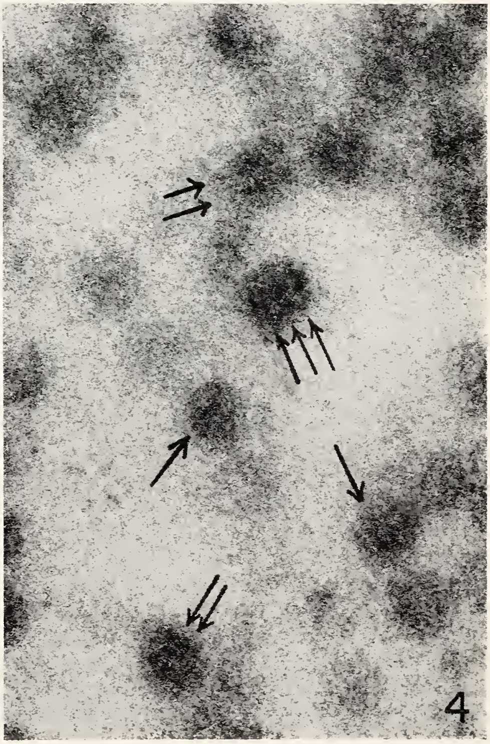
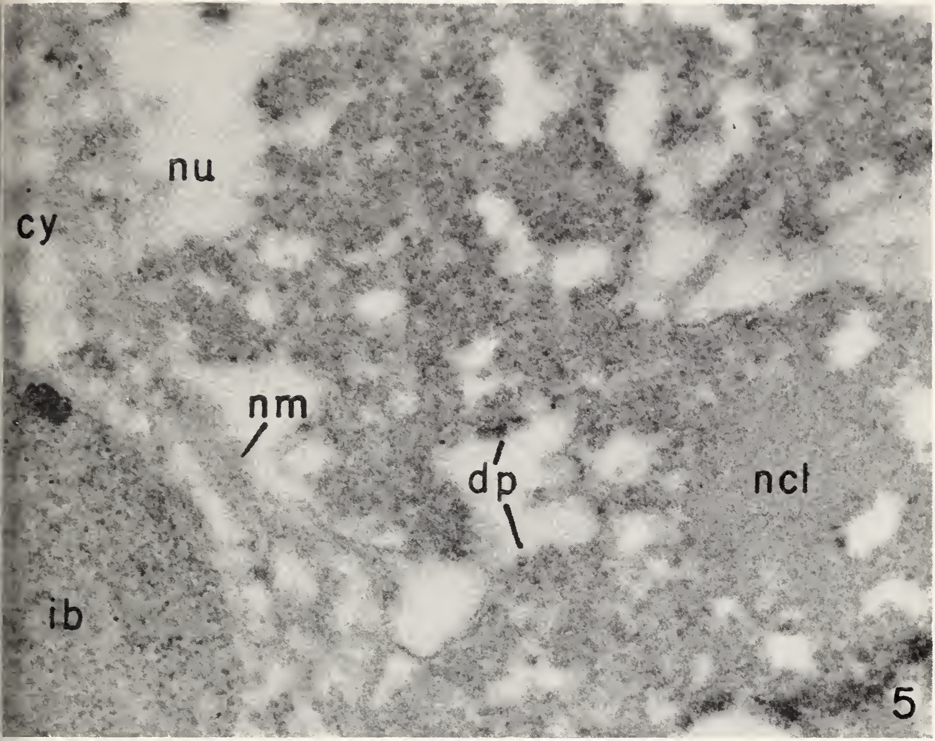

4

Plate 51

Figure 5.—Portion of leukemia tumor cell showing aggregates of dense intranuclear particles (dp). Individual particles seem to be associated with nucleolar material (ncl). Nuclear membrane (nm) between nucleus (nu) and cytoplasm (cy), and portion of large "inclusion body" (ib) are indicated. × 120,000

Figure 6.—Cell similar to that of figure 5. Intranuclear particles (dp) appear to be related to filamentous material (f). Nuclear membrane (nm) located at *right.* × 120,000

Figure 7.—Portion of leukemia tumor cell. Nucleoplasm close to nuclear membrane (nm) discloses aggregates of filamentous material (f) and rows of tiny dense particles connected by fine filaments (*arrow*). × 120,000


nu
cy
nm
dp
ncl
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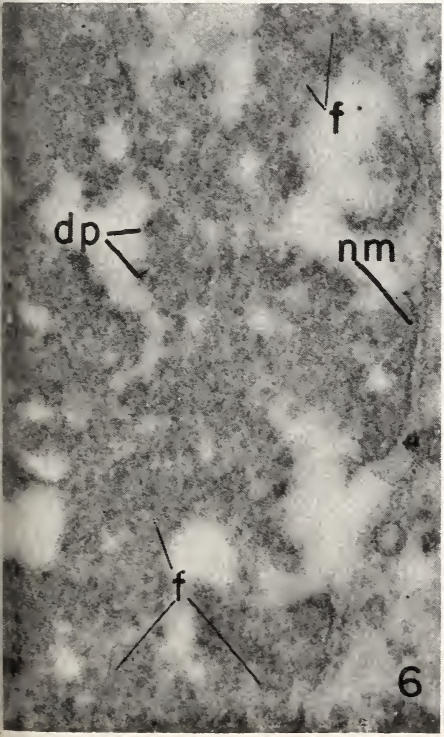
5



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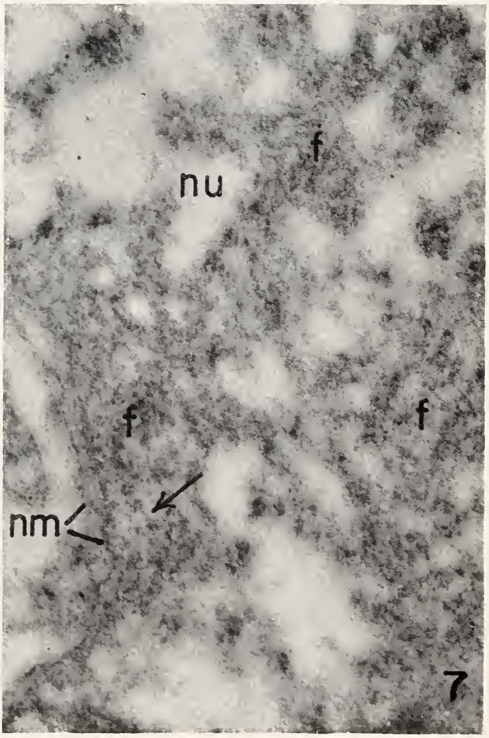

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Plate 52

Figure 8.—Cytoplasmic area of polyoma tumor cell containing normal mitochondria (mi) and transforming mitochondria with densely outlined membranes possessing dense (mi_2) or faint (mi_3) matrix but no cristae. Inclusion bodies (ib, ib_3) are limited by a double membrane and show matrices of varying density and structure, within which dense particles may be found (ib_2). Aggregates of viruses (v) also seen, both in remainder of "inclusion body" (*upper right*) and freed from disrupted inclusion body (ib_1) in which double membrane is observed (*lower left*). RNP granules appear throughout cytoplasm. × 60,000

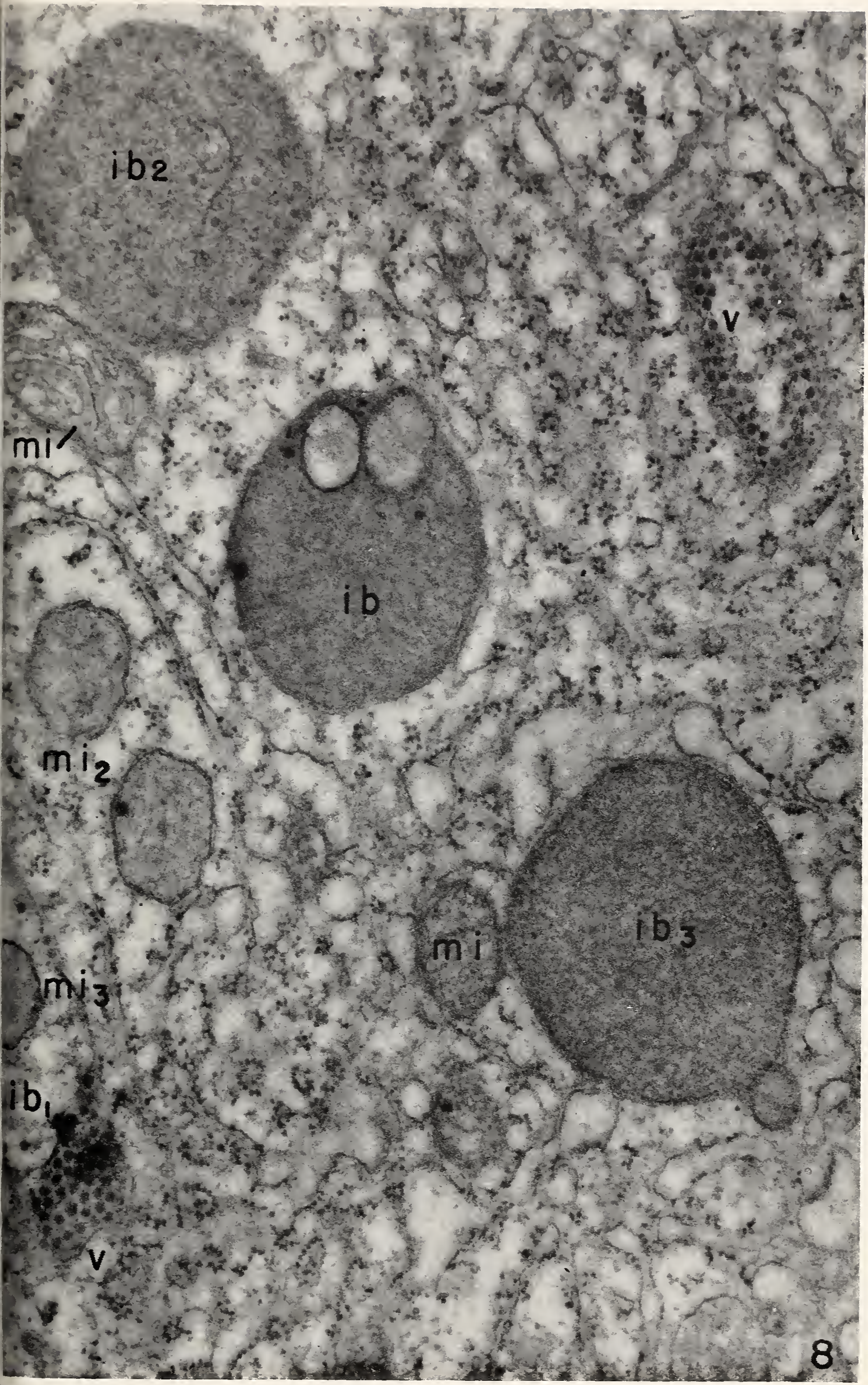

ib2
v
mi
ib
mi2
ib3
mi
mi3
ib1
v
8

Plate 53

Figure 9.—Portion of cytoplasm of leukemia tumor cell showing various "inclusion bodies" (ib), some of which are limited by distinct double membranes. In their matrices, arrays of fine filaments and rows or aggregates of tiny dense particles (p), and occasionally large and very dense particles (p_2), are seen. At *upper* part is a presumably transforming mitochondrion (tmi), within which are observed arrays of filaments and dense tiny particles, and uniform, regularly arranged vesicles. The latter are presumed to represent fragmentation stages of mitochondrial cristae. In *center* another body shows filaments (fi) possessing a very fine axial period. Fragments of such types of mitochondria (*arrows*), lipide clumps (l), and RNP granules are found throughout cytoplasm. × 120,000

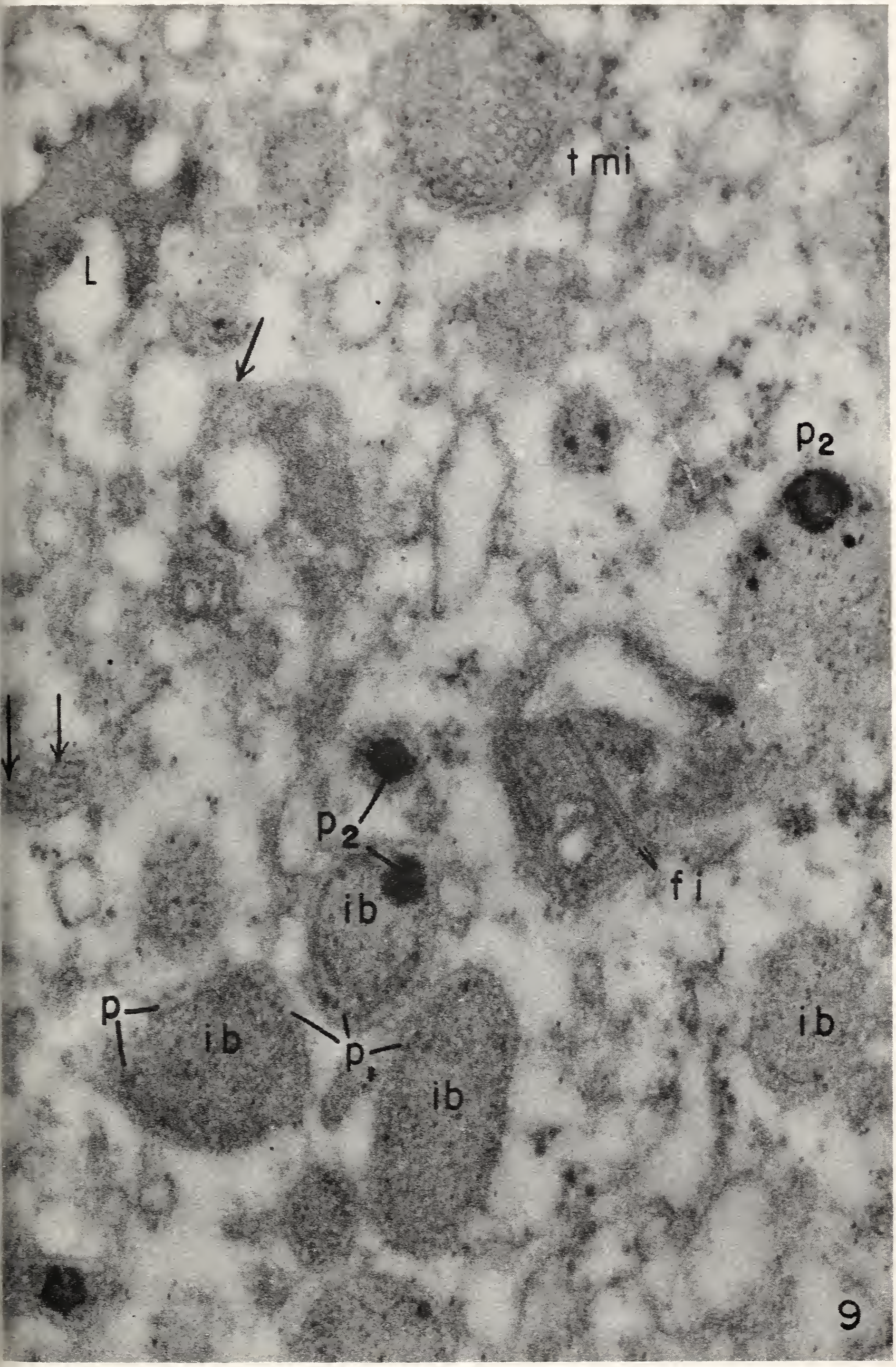
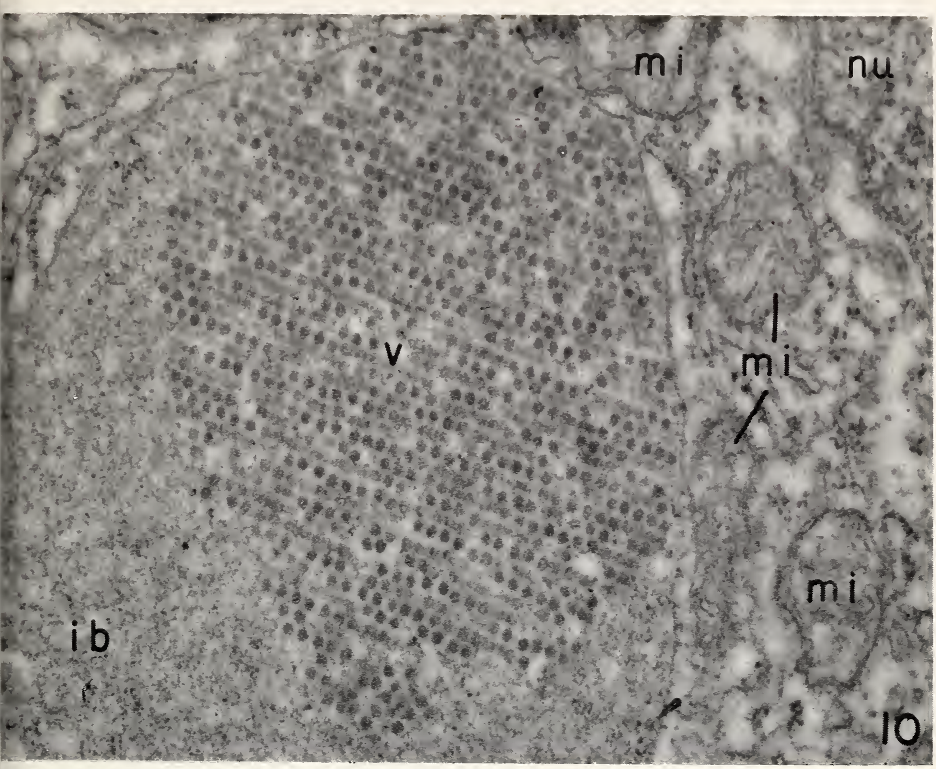

tmi
L
p2
p2
fi
ib
p
ib
p1
ib
ib
9

PLATE 54

FIGURE 10.—Detail of inclusion body (ib), in cytoplasm of polyoma tumor cell, showing crystalline array of viruses (v). At *right,* mitochondria (mi) and a portion of nucleus (nu) are seen. × 60,000

FIGURE 11.—Portion of leukemia tumor cell showing, at border, plasma membrane (pm) and peripheral cytoplasm composed of fine filamentous material (f). *Note* strand of apparently same material (f_1, f_2) through cytoplasmic matrix, down to nuclear membrane region (nm). Mitochondrion (mi), numerous free or aggregated RNP granules, and rough profiles of the endoplasmic reticulum (er) are also seen. × 120,000


mi
nu
v
mi
mi
ib
10

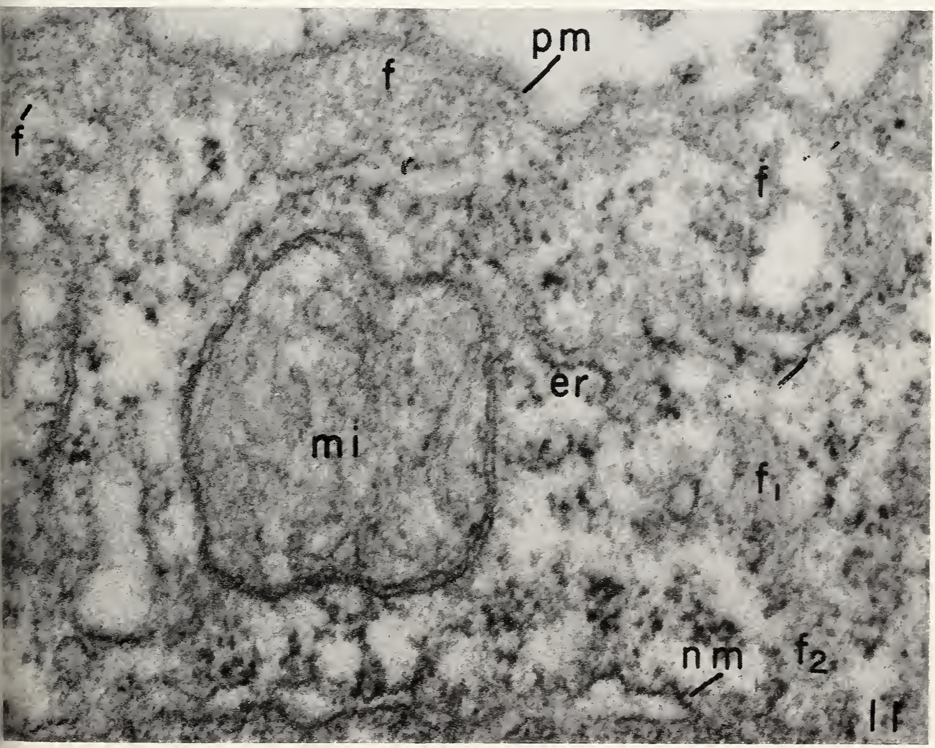
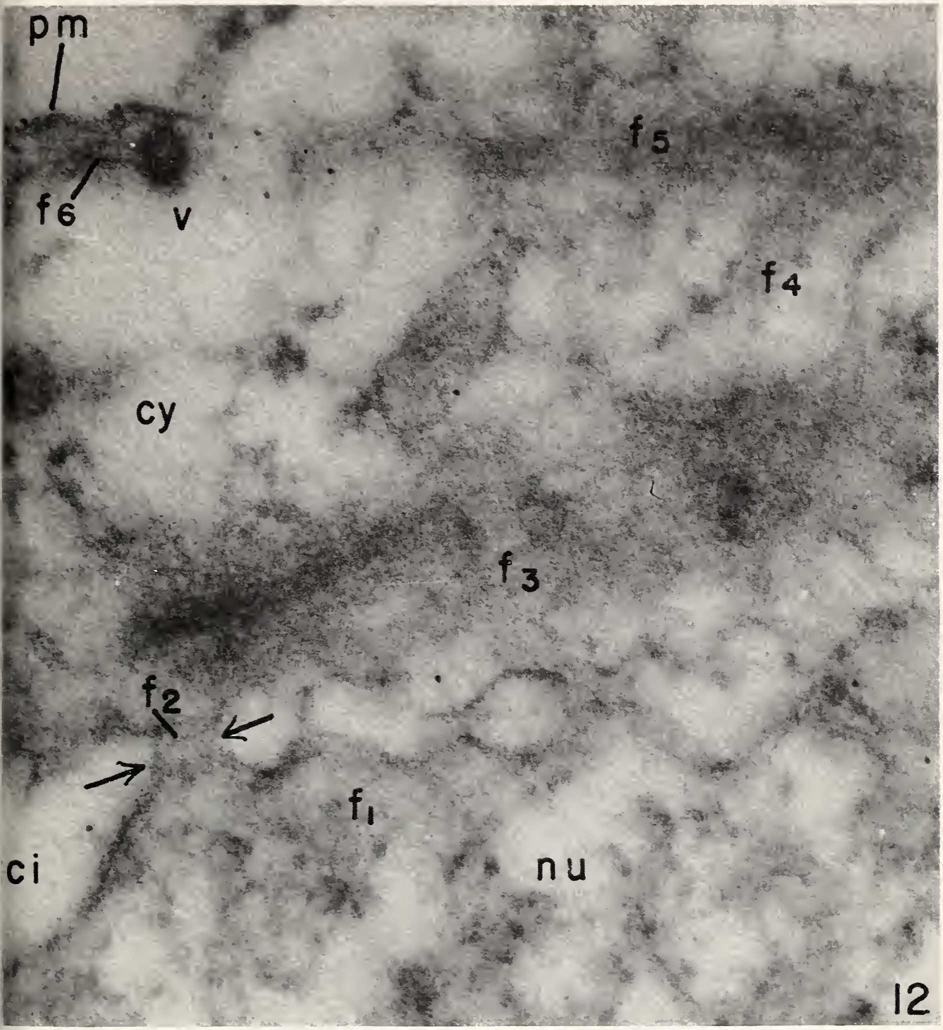

pm
f
f'
f
er
mi
f₁
nm
f₂
11

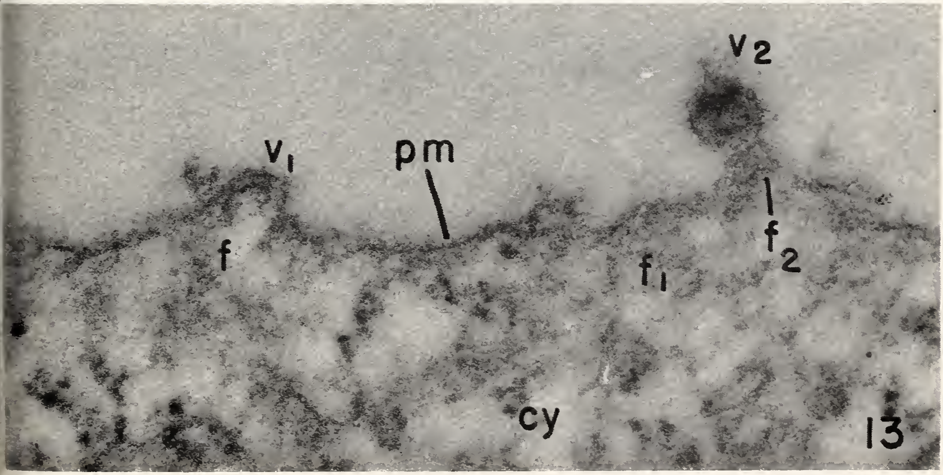
PLATE 55

FIGURE 12.—Portion of leukemia tumor cell showing nucleus (nu), cytoplasm (cy), nuclear envelope with perinuclear cisterna (ci) interrupted by numerous pores (*arrows*). Unoriented fine filaments are seen in nucleoplasm (f_1). In pore channel (f_2) and on cytoplasmic side of pore, filamentous material (f_3) appears in parallel array. Occasionally strands of perinuclear filaments (f_4) are seen to be continuous with the cortical cytoplasmic filamentous material (f_5, f_6). Virus particle (v) shown at cell border (pm). *Note* its relation to peripheral cytoplasmic filaments. × 120,000

FIGURE 13.—Leukemia tumor cell showing accumulated filamentous material (f, f_1) at subplasmalemmal cytoplasmic region. Plasma membrane (pm) and fine filaments enter in virus particle formation, as in early stage (v_1), and in more mature form (v_2), where filamentous material (f_2) is seen in direct relation with virus particle. × 140,000


pm
f6
v
f5
f4
cy
f3
f2
f1
ci
nu

12


v2
v1
pm
f
f1
f2
cy

13

PLATE 56

FIGURE 14.—Region between two leukemia tumor cells. Four virus particles (v) are seen in interstitia, sometimes appearing to be connected with plasma membranes (pm) (*arrow*). "Inclusion body" (ib) shows a filamentous matrix with rows of tiny dense particles. Lipide clumps (l) and normal mitochondrion (mi) are also seen. × 120,000

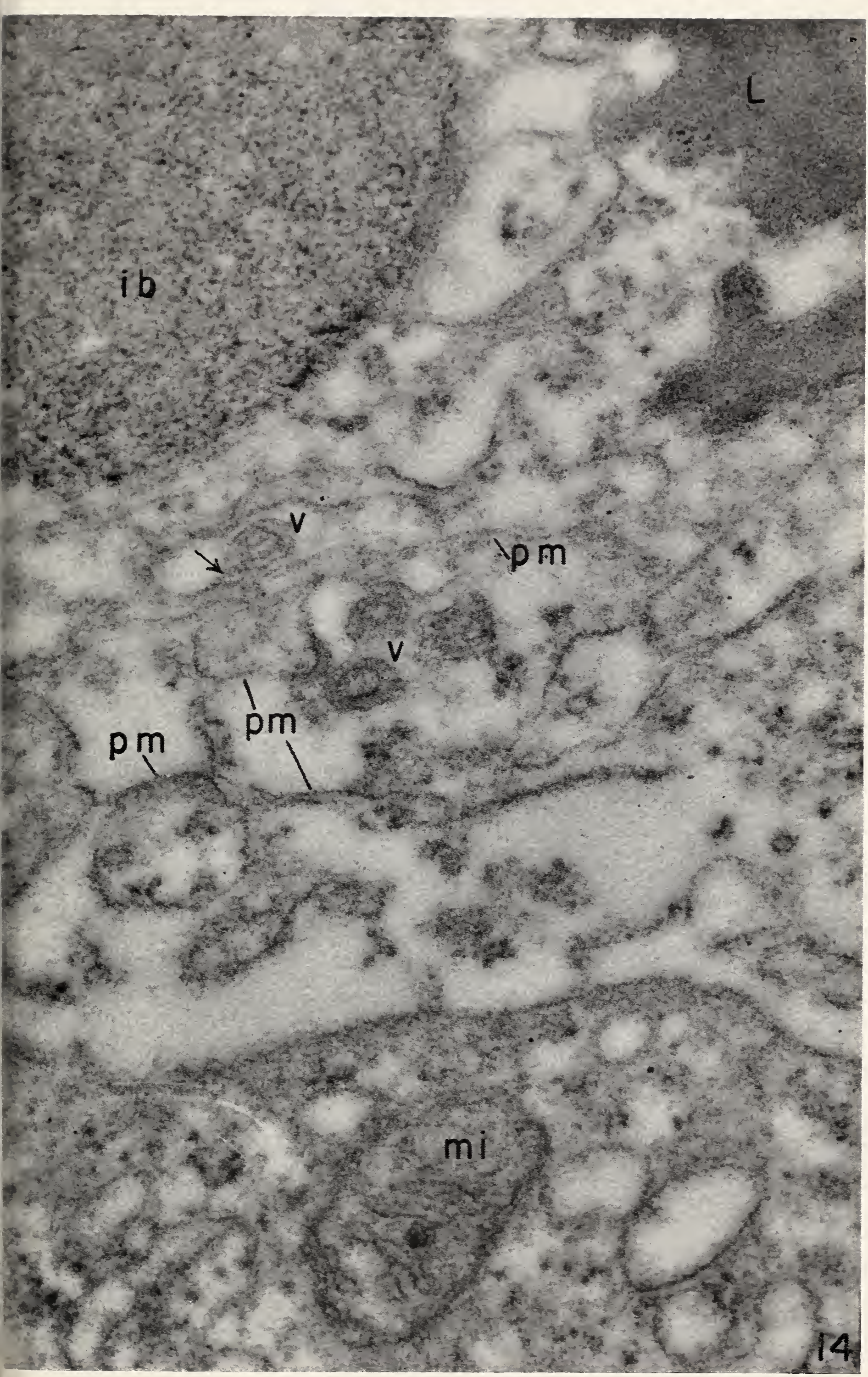
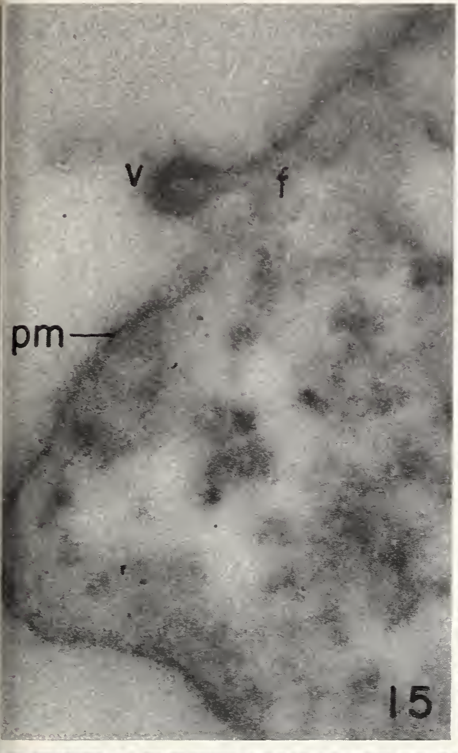

L
ib
v
pm
v
pm
pm
mi
14

Plate 57

Figure 15.—Edge of leukemia tumor cell showing virus particle (v) attached by long connection to plasma membrane (pm). Fine filamentous material (f) from cortical cytoplasm is seen to be continuous with virus attachment. × 100,000

Figure 16.—Periphery of leukemia tumor cell showing infoldings of plasma membrane (pm), partially formed virus particle (v_1), and more advanced particle (v_2) in which cross striations can be seen in virus coat. × 80,000

Figure 17.—High magnification of newly formed leukemia virus particle. *Note* complex membrane and spiral or coiled internal array of filamentous material which appears to connect with plasma membrane (pm). × 440,000



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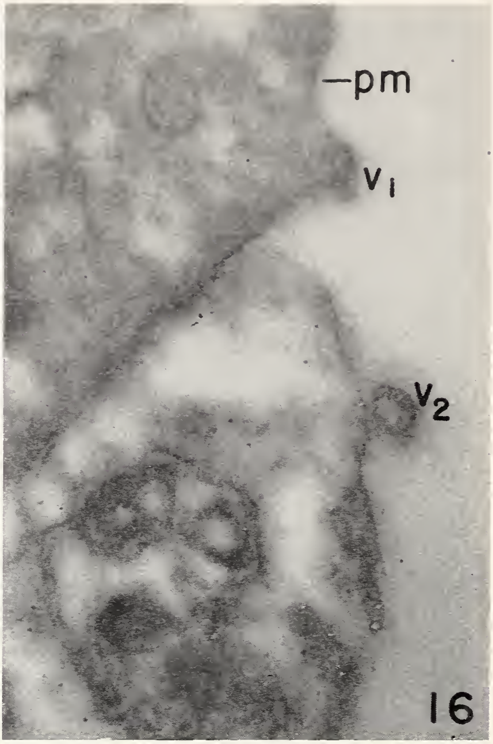

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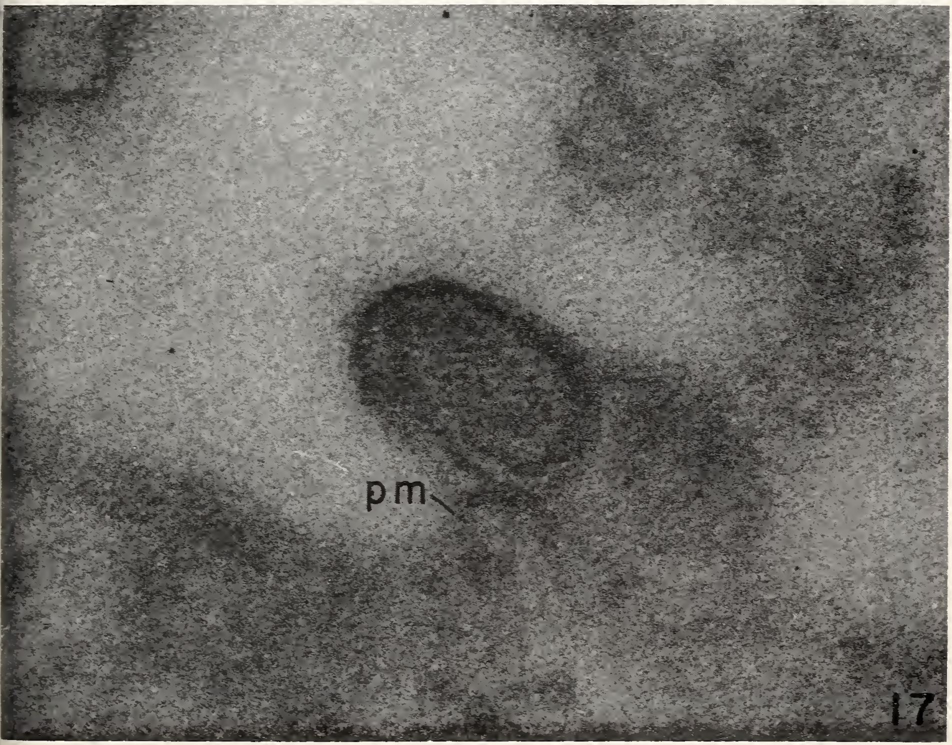


17

Plate 58

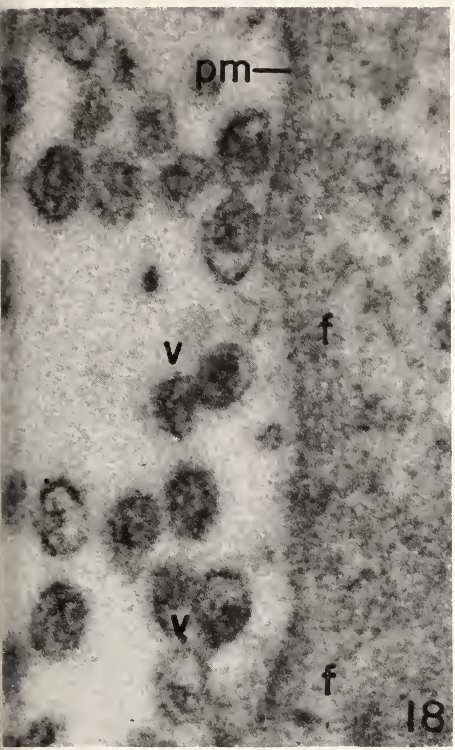
Figures 18 through 21 show mature and young leukemia-type viruses found at borders of cells of a polyoma parotid tumor (*cf. 3*).

Figure 18.—*Note* parallel arrays of fine filaments along edge of cell. Virus particles (v) are seen outside plasma membrane (pm) in intercellular space. × 120,000

Figure 19.—Similar to figure 18 but displaying a half-formed virus particle (vi) and its relation to plasma membrane (pm) and to fine filamentous material (f) in cortical cytoplasm. × 120,000

Figure 20.—Fine filaments (f) in peripheral cytoplasm are near where a maturing particle (v_1) is broadly attached to plasma membrane (pm). × 120,000

Figure 21.—Viruses already detached from plasma membrane (pm) and possessing distinct coats (vc) with cross striations (cs). *Arrow* indicates probable presence of pore. × 140,000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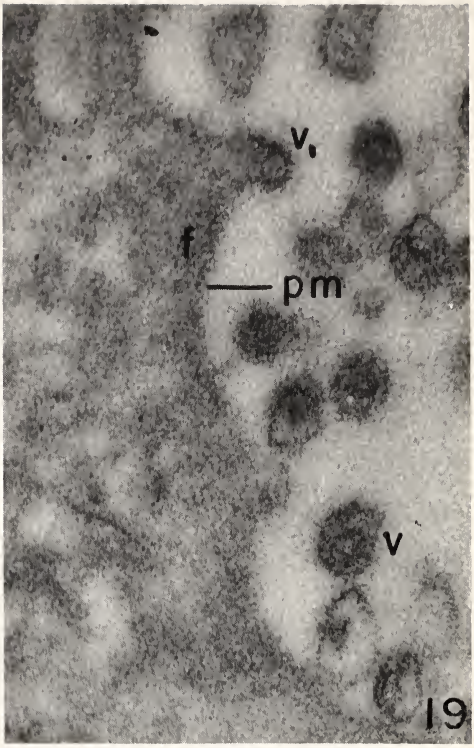
18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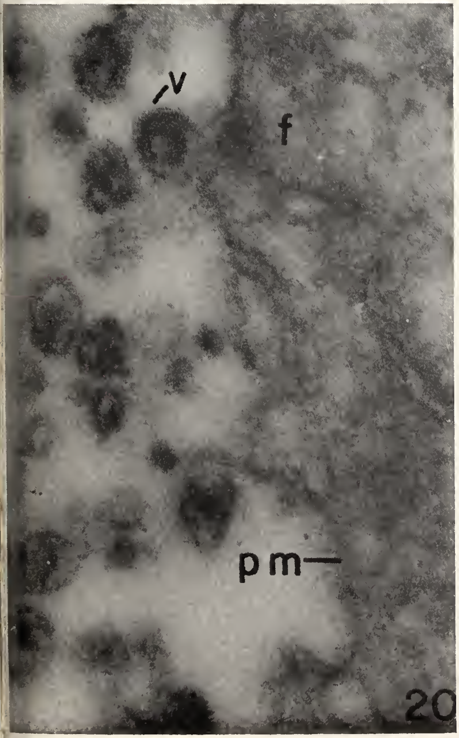
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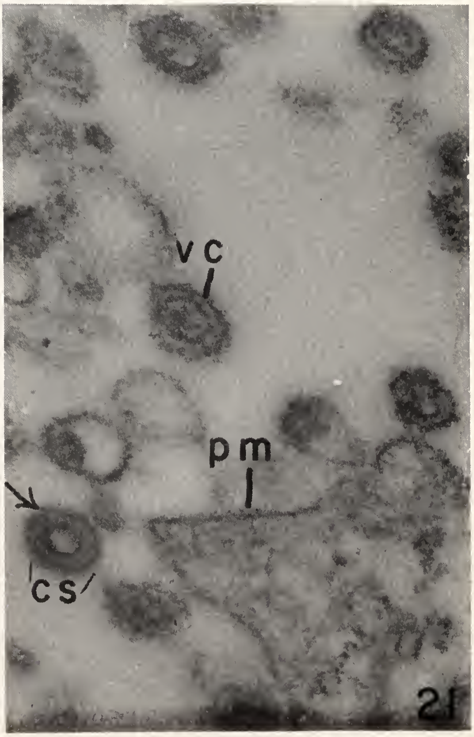
vc
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21

Cancer Symposium

Sponsored by
American Chemical Society, Washington, D.C.
Held September 16, 1959
Atlantic City, New Jersey

Edited by
Joseph Leiter

Present Status of Studies on Tumor-Producing Viruses[1,2]

JEROME T. SYVERTON, ***M.D., Department of Bacteriology, University of Minnesota, Minneapolis, Minnesota***

THIS will be a brief review on the relationship of viruses to carcinogenesis. Although I am a virologist with continuing interest in virus-induced tumors, I am not trying to advance the virus theory of cancer. The question is not whether *all* tumors are caused by viruses but *what* tumors are so caused, and how.

Viruses and cells are inseparable associates. Modern study of viruses has been and continues to be responsible for vast strides in the understanding of human disease. Before 1950, only 21 human viral diseases were recognized (*1*). In a single decade since, more than 150 hitherto unknown viruses have been characterized, and many others under study are known to be distinct from established types (*1*, *2*). This amazing accomplishment reflects the development of a new discipline, cell culture. Cell cultural methods, technologically improved since 1950 (*3*), have turned up a host of viruses in the human environment. The human respiratory tract, orointestinal tract, genitourinary tract, and even the viscera have harbored unsuspected viral nonpathogens, opportunist pathogens, and viruses newly associated with particular diseases.

I wish to consider some particular aspects of viruses, cells, and cancer as my associates and I at the University of Minnesota see the problem. We believe that understanding disease at the cellular level (*4*, *5*) is prerequisite to understanding and treating it at the level of the intact host.

What is a virus?—Most people ask whether a virus is "alive." This is not an idle question, because we must have some idea what a virus is, apart from the way in which it is identified. Perhaps we should first ask: What is a cell? A cell is a natural device for carrying out certain biochemical syntheses and for building replicas of itself. To permit this accomplishment, it incorporates a built-in code of instructions, the genic-chromosomal apparatus. The virus likewise has built in it a code of instructions, in the form of nucleic acid wrapped as a package to facilitate entry into a susceptible host cell. This concept of virus involves two key points: (*a*) foreignness to the cell, implied by the fact that the nucleo-

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[1] Presented at the Cancer Symposium, American Chemical Society, Atlantic City, N.J., September 16, 1959.

[2] Aided by grants from the American Cancer Society, Inc., the National Foundation, and the National Cancer Institute, National Institutes of Health, Public Health Service.

protein code includes instructions for specific packaging, and (*b*) complete dependence of the virus on the cell for physiologic activity. This dependence results from absence of a mechanism within the virus capable of independent fulfillment of the instructions incorporated in its nucleoprotein code. This concept readily differentiates transforming agents and cellular genes from the category of virus. The virus is a relatively passive partner in a peculiar host-parasite relationship. It is a packaged blueprint that causes a cell to divert its energy partly or wholly to the manufacture and packaging of more virus. Incidentally but as a result the cell is physiologically impaired or destroyed. We call this the cytopathogenic effect of a virus. We do not know whether this impairment is a direct or indirect consequence of virus production. Impairment might be attributed to: (*a*) deleterious toxic influence of newly replicated virus particles as they accumulate within the infected cell; (*b*) a simple inadequacy of the cell to bear the combined load of cell maintenance and virus manufacture; and/or (*c*) the diversion of essential metabolic processes from cell maintenance, the natural economy of any cell, to virus production. Despite this concept of virus as a packaged code, for purposes of classification viruses are accepted as parasitic microorganisms. Is a virus "alive"? Present concepts of viruses indicate why the question has no real answer. The transition from "alive" to "not alive" has no discontinuity. From the foregoing consideration, it is apparent that the infected cell-virus complex in a sense represents an entity, a new microorganism. Any virus produced by this complex therefore is a reflection of the complex, a cell product. Cells cannot be regarded simply as media. Cells are intricate factories which in the body are arranged as intricately organized tissues. Cells under direction of virus function vicariously and/or they are destroyed.

Virologists now have at their disposal primary cultures of cells taken directly from tissues, stabilized strains of cells in continuous culture, and purified continuously propagated strains derived from single cells. A major contribution to medical research has been the evolution of mass preparation procedures. For example, at Minnesota we employ weekly from 3,000 to 5,000 cell cultures prepared from 31 cell strains of human, monkey, horse, cow, pig, domestic and cottontail rabbit, and mouse origin. Development of culture technique is continuing, in search of improved means for characterizing animal cells and production of cells of desired characteristics. One member of our group (*6*) has developed use of an enzyme, collagenase, for better disruption of tissues as a source of cells, so as to obtain primary and continuous cultures of pulmonary and other body cell types. Another member of the group (*7, 8*) has devised a hemagglutination test for species specificity of cultivated cells, so that now we can at least verify the identity of cells originally derived from different animals. Studies of cellular chromosome morphology and number, and of the physiologic activities of cells, are being pursued (*4*). The ultimate objective is to propagate cells of known type continuously from body tissues with normal chromosome constitution and defined relation to cells *in vivo* for comparative studies of cancerous cells of similar origin.

Viruses and cancer.—The relationship of viruses to cancer in the past has been clouded by emotional factors. Today our attitudes are not free of emotion, but do have a more rational basis. Cancer concerns you and me as individuals and as practitioners of medicine or research. At present, 1 of 4 or 5 of us in this room can expect to have cancer, and there is every reason to believe that, as the incidence of other diseases is reduced and the lifespan prolonged, that proportion will rise. Although we do not yet know that a single human cancer is caused by a virus, we are vitally interested in viruses as the causes of human cancer. Studies of experimental cancer and the existence of a large number of known tumorigenic viruses of animals support the belief that at least some human tumors must be similarly incited.

In a discussion of the cause of cancer, it is not unusual for authorities to state that there are many kinds of cancer and that cancer therefore must have many different causes. This view is conditioned by the observation that cancer can develop from any of a wide range of cell types located at any part of the body and exposed to widely assorted environments. The pathologist recognizes a sameness in the spectrum of cancer as shown by the same general defects, by progression in autonomy and by the assumption by malignant cells of an embryonal morphology or "anaplastic" appearance. Capacity or ability of a pathologist to recognize changes becomes intuitive. A conjunction of observations has led many investigators to consider that cancer cells result by "somatic mutation," induced by one of a number of possible factors. Although cancer researchers categorize "causes" of cancer, it is more helpful to consider the factors in the disease process simply as exciting, mediating, operating, and modifying. For instance, diphtheria is excited by a bacterial virus (*9–11*), mediated by *Corynebacterium diphtheriae*, operated by a bacterial exotoxin, and modified by the reaction of the host. We have good reason to believe that cancer is a disease modified by the host. We know that it is operated by altered body cells. Since experimentally it can be shown that the altered cells exhibit heritable properties, at some stage in the evolution of a tumor the disease is mediated by cellular genetic constitution. This view directs emphasis away from the categories of "causes"; what we want to know is how the various factors interact.

We know that a lengthening list of viruses is involved in the etiology of animal tumors, as shown in table 1. The exciting agent of each of these tumors is a specific virus separable from all others. Nine of the neoplastic diseases result immediately or finally in unequivocal malignancy. Contrariwise, 8 of the viruses collectively induce a broad spectrum of tumors that do not satisfy all criteria of malignancy. As you see, this list of tumorigenic viruses has grown since 1951. Much new information came from use of newborn animals in 3 laboratories. In 1951, Gross (*12*) showed that cell-free extracts prepared from leukemic

Table 1.—Viral tumors of animals

1908	Fowl leukemia (Ellerman and Bang)*
1911	Fowl sarcoma (Rous)*
1920	Bovine papilloma (Magalhaes)
1932	Canine oral papilloma (DeMonbreun and Goodpasture)
1932	Rabbit fibroma (Shope)
1933	Rabbit papilloma (Shope)
1933	Fowl lymphoma (Furth)*
1936	Rabbit oral papilloma (Parsons and Kidd)
1936	Mouse mammary carcinoma (Bittner)*
1938	Frog kidney carcinoma (Lucké)*
1951	Equine cutaneous papilloma (Cook and Olson)
1951	Mouse leukemia (Gross)*
1953	Mouse parotid tumor (Gross)*
1953	Squirrel fibroma (Kilham, Herman, and Fisher)
1955	Deer fibroma (Shope, Mangold, MacNamara, and Dumbell)
1956	Mouse leukemia (Friend)*
1957	Polyoma (Stewart and Eddy)*

*Neoplastic virus disease which becomes malignant.

tissue from mice of a strain with a high incidence of spontaneous leukemia (AKR) would, when injected into newborn mice of the C3H (Bittner) strain with low leukemic incidence, produce leukemia months later in the injected mice. The induction of leukemia could be repeated with extracts of leukemic tissues of the injected C3H mice. These results have been confirmed abundantly. Next, Gross (*13*) and Stewart (*14*) reported independently that injection of newborn mice with an extract of leukemic tissue resulted in development of parotid-gland carcinomas and/or fibrosarcomas. This work added notably to the list of mammalian tumor viruses initiated in 1936 by Bittner (*15*), with the demonstration that an agent with all properties of a virus could produce mammary cancer when transferred to newborn mice at their first feeding. Recently, Stewart and her associates (*16*) have transferred cell-free extracts from leukemic mice to tissue cultures, and after repeated passage in culture, harvested an agent responsible for induction of a variety of solid tumors in recipient mice. This "polyoma" virus likewise must be injected into newborn mice. These 4 instances have demonstrated unequivocal association of a virus with a malignant tumor or tumors. Bittner (*17*) and Mühlbock (*18*) have emphasized hormonal influences rather than virus as the "direct" cause of the mouse mammary cancer. This distinction would, in my view, be quite significant if it were demonstrable that the tumor cells never contained virus. It is inconceivable that virus might infect hormone-producing cells in the body, and so impair or modify their function that carcinogenic hormones were produced. But since virus is shed into the milk, and in many instances is recoverable in high titer from the mammary tumors, the distinction is not readily substantiative. According to my earlier review of the possible origin of cancer, there is room for viruses, hormones, and many other factors in etiology. Our primary need is not to decide which is causative, but how each is causative.

Dependence on newborn animals for demonstration of etiologic activity of these tumor viruses suggests that the development of cancer or leukemia under ordinary conditions reflects the inability of an animal's immune mechanisms to resist an agent encountered neonatally. The unborn or newborn animal is unable to generate immunity to a wide variety of agents, living or otherwise. Since constitutional defect of the newborn does not explain susceptibility to a neonatally encountered agent in later life, it has been theorized that the neonatal encounter results in a continued state of specific unresponsiveness to the particular antigens contained in the agent. That is, "actively acquired tolerance" ensures that particular neoplastic diseases can be transmitted from parent to progeny, but not from the individual in mature life. It does not appear that such tolerance need be absolute. Gross (*19*) showed that tissue extracts of normal C3H mice produced a significant number of parotid tumors in C3H mice inoculated when newborn. Here the important factor in the development of parotid tumors was not the presence of agent in recipient mice, for both donor and recipient mice had that; the significant difference was the inability of newborn mice ultimately to resist added virus encountered during the first days of life. The difference could be explained by acquired immunological tolerance. Woolley (*20*) provided other evidence to support this suggestion when he found that normal mice given sufficient cortisone to depress immune response also developed parotid tumors. Kaplan (*21*) showed that a variety of mice with a low natural incidence of leukemia, the C57BL strain, developed leukemia of thymic origin after fractional X irradiation. If thymus gland from an unirradiated C57BL mouse was transplanted into a thymectomized preirradiated C57BL mouse, leukemia again developed from cells of the unirradiated transplanted thymus. If, however, the thymectomized irradiated mouse was given C57BL mouse bone marrow before transplantation of normal unirradiated thymus gland, leukemia usually did not develop. In these experiments, since leukemia developed from unirradiated cells, it could not be explained as a result of somatic mutation by mutagenic action of X rays. Development of leukemia in the mice given sufficient irradiation to destroy the immune response is explained most readily by the inability of the irradiated animal to resist a tumor agent introduced with the unirradiated thymus cells. Protective action of injected bone marrow would be expected, since it is known that such injection may repair capacity for immune response of irradiated hosts through repopulation of damaged bone marrow. These and other experiments demonstrate that tumor viruses may exist as temporary residents of animals without necessary induction of tumors or leukemia, though the viruses are demonstrably capable when introduced into immunologically inadequate hosts. A tumor virus even may be transmitted from generation to generation, vertical transmission, without manifestation of its presence.

I have departed from the cellular view of virus-induced neoplasia to emphasize these grosser aspects of virus-host relationship, since these aspects are involved in an understanding that cancer can be noninfectious in the usual sense and still be induced by a virus. On the basis of our present knowledge of viruses and on the assumption that host conditions are appropriate for manifestation of virus infection as a tumor or cancer, three alternative results of infection can be conceived: (*a*) Infection results in an altered cell that continues to produce virus. (*b*) Infection produces an altered cell, but does not permit virus reproduction, and (*c*) infection results in the production of altered virus with eventual destruction of the producing cell. In other words, is virus production compatible with cell proliferation, or if it is not, how are the two processes reconcilable? Two biological phenomena may provide clues. The first is the phenomenon of *lysogeny* (*22*). I have said that a virus is a code of instructions wrapped in a package. Certain bacterial viruses, upon loss of the protein coat and delivery of the nucleic acid into the bacterium, do not undergo replication of the nucleic acid or instruction code. The viral code becomes associated with the cell code, *i.e.*, with the cell genic chromosomal structure. Spontaneously at intervals, or purposefully under the influence of an activating agent such as irradiation or nitrogen mustard, the preserved bacterial code, or provirus, detaches from the cell chromosomal structure to undergo replication and repackaging. The result is virulent, destructive virus that lyses the host bacterium and may infect and lyse other susceptible cells. On encounter with insusceptible cells again, the infecting virus re-enters the provirus state. While in this state, the virus code is undetectable as virus, and is treated by the bacterial cell as a portion of its chromosome. The code is replicated and transmitted to daughter cells just like native cell code. The second phenomenon is the *infectivity of viral nucleic acid*. The studies to which I refer were carried out in collaboration with Holland and McLaren (*23–26*). We reported, for example, that type 1 poliovirus will infect only cells of primate origin, and that this susceptibility of primate cells depends on an interaction between the protein package of the poliovirus and lipoproteins of the cell surface. Nonprimate cells are quite insusceptible to poliovirus because, lacking the cell-wall receptor, they cannot adsorb the virus. We also showed that nucleic acid (the viral instruction code) extracted with phenol from type 1 poliovirus, Coxsackie A-9, Coxsackie B-1, and ECHO-8 viruses infected ordinarily insusceptible nonprimate cells. This infectivity of the viral RNA was manifested on insusceptible cells in established continuous culture, in primary monolayer culture, on suspensions of minced tissues, and on living animals inoculated intracerebrally. Cells of rabbit, swine, mouse, guinea pig, chicken, and hamster origin could be infected equally well with enterovirus RNA. Each of the viruses so produced was identical to the virus donating the RNA. Virus produced from infecting RNA was neutralized by homotypic antiserum, resistant to ribonuclease treatment, and insusceptible to adsorption or replication by nonprimate cells, even of the strain producing the virus from RNA. In RNA-infected cell cultures, virus was produced in a single cycle, without overt cytopathic effect on nonprimate cells or disease in intracerebrally inoculated animals.

Generalizing from these two phenomena, one can suggest that viral nucleic acid, freed of its protein overcoat under influence of any of a variety of agents, could infect any mammalian cell which it encountered, and either replicate more of the virus from which it was derived or lysogenize the recipient cell. Production of infectious virus from RNA would not entail destruction of the cell population. The lysogenic state might be abrogated years later under influence of a provocative agent such as the chemical carcinogen or radiation. These possibilities permit us to accept any of the alternatives for tumor virus-cell interaction postulated previously. As yet, we do not know whether we should associate a proliferative effect on cells with the state of apparent or inapparent infection. We do know that considerable latitude of postulation is permissible, since tumor viruses all do not act with equal speed. Rous sarcoma virus induces a fully malignant tumor in a few weeks. Rabbit skin carcinoma, in contrast, results after many months, by development from a clearly defined, preceding benign papilloma. A virus may initiate neoplasia by inducing cell proliferation, and/or increasing genetic variability of cells. To investigate these questions, we are handicapped by not having available a biological test for detection of intracellular proviruses, or a cell-culture test for cytomalignancy comparable to the cytopathogenicity test used for the study of enterovirus infection. To emphasize our need and the importance of these tests, I may remind you that it was the use of trypsin to disperse cells (*27*), a comparatively simple laboratory tool, that led to monolayer cultures (*27*), quantitative assay of animal virus particles by plaque count (*28*) and clonally derived mammalian cells strains (*29*) that led to the discovery of more than 100 new viruses that undoubtedly had existed and been productive of disease in man's environment for centuries.

This has been more a speculative than a definitive lecture on viruses. Perhaps its major lesson is that with virology as in other areas of medical science, we must get from the patient to the laboratory before we, confident of treatment or prevention, can return from the laboratory to the patient.

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Induction of Tumors *In Vitro* With Viruses[1]

R. DULBECCO, ***Division of Biology, California Institute of Technology, Pasadena, California***

This paper has three main objectives: (1) to examine the conditions under which a virus, able to produce malignant tumor in the animal, can transform *in vitro* normal cells into characteristically altered cells, different from the normal cells in morphological and physiological properties; (2) to discuss the nature of the virus-cell interaction leading to this transformation; and (3) to investigate the relationship of the virus-transformed cells to cancer cells as they are found in the animal. For these purposes a single virus-cell system will be used, that constituted by the Rous sarcoma virus and by cells derived from the chicken embryo, a system extensively used in our laboratory.

We shall briefly consider the characteristics of this model system. The virus is constituted by particles about 80 mμ in diameter, containing ribose nucleic acid, protein, and lipides. Virus stocks can be easily obtained by extracting the fully developed Rous sarcoma in chickens, or by collecting the supernatant of tissue cultures of chicken-embryo cells infected by the virus. The cell-free filtrate thus obtained has the ability to infect young chicks, and to reproduce in them the characteristic sarcoma. The infectious quality of the virus is considerably heat-labile, being lost at 37° C. in the usual media with a half life of 2½ hours.

The virus is propagated *in vitro* in cells derived from chicken embryos; turkey-embryo cells would also be suitable. One important problem arises in the choice of a suitable source of embryos. It is known that of the many available breeds of chickens some are considerably resistant to the Rous virus; this resistance appears to be of a genetic nature, and is manifest also in the cell cultures derived from embryos of these breeds. A genetically sensitive breed must therefore be chosen; this can be accomplished only in an approximate way, since there are no completely inbred strains of chicken in this country. A number of poultry farms maintain relatively inbred stocks, some of which are satisfactory for work with the Rous virus. These stocks, however, are not entirely uniform in genetic constitution and contain a fraction of relatively resistant animals. Since cell cultures are usually produced in batches from randomly chosen populations of embryos, they usually contain a proportion of genetically resistant cells. The proportion of genetically resistant cells is generally small and does not cause important difficulties. If needed, cultures entirely constituted by genetically sensitive cells can be obtained, by making cultures from single embryos.

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[1] Presented at the Cancer Symposium, American Chemical Society, Atlantic City, N.J., September 16, 1959.

The cell cultures sometimes show, in addition to this type of resistance, another type of resistance to the Rous virus, of a physiological nature. Physiological resistance may be caused by some conditions of cultivation of the cells, such as age of the cultures, composition of the medium, and density of the cultures; the relative importance of the various conditions and their significance in causing physiological resistance are unknown.[2]

The cultures generally used with the Rous virus are monolayer cultures, in which the cells sparsely cover the glass bottom of a petri dish. A standard type of monolayer culture is obtained from decapitated 10-day chicken embryos dispersed in trypsin; these cultures contain mostly elongated cells, described as fibroblasts, deriving in majority from the body wall. For special purposes, cultures can be prepared either from trypsin-dispersed hearts of 10- to 16-day embryos—referred to as heart fibroblasts—or from lungs of 16-day embryos, also dispersed by trypsin. The lung cultures contain, in addition to fibroblast-like cells, many epithelial cells, which can be enriched by proper techniques. For best results sparse, young cultures, usually secondary 1-day-old, are used.

We want now to examine the effects of the exposure of suitable cultures to the Rous virus. Several effects are observed; they will be examined in succession.

The most noticeable effect is a morphological one. It was observed by Manaker and Groupé (*1*) that suitable cell cultures exposed to a virus preparation containing a small number of infectious units develop foci of characteristic round cells. This phenomenon by which an elongated cell, upon infection with the virus, becomes spherical will be defined as "conversion."

Subsequent study by Temin (*2*) showed that conversion of elongated cells to round morphology is only a special case of conversion. Many other types of morphological conversion caused by the Rous virus were observed. In general we can say that the morphological cell type deriving from the interaction of chicken cells *in vitro* with the Rous virus depends on two factors at least: the type of virus and the type of cell used.

We shall examine the types of conversion observed as a function of the two main variables, the virus and the cell type.

We shall first consider the case in which identical cells are infected by different virus types. Populations of identical cells can be obtained as progeny of a single cell: They are called clonal populations. Different virus types can be obtained by the selection of viral variants in standard virus stocks; the variants are selected because they convert cells to morphological types different from the standard ones. Several variants of this type have been found thus far. When clonal populations of fibroblast-type cells are exposed to different variant types of virus, different types of conversion are found. The two extreme cases are the conversion to a round, highly refractile cell type, which is the one produced by the standard virus, and the conversion to an extremely elongated cell type. Other virus variants convert the cells to intermediate types.

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[2] *Footnote added in proof:* It has been recently shown by Rubin (personal communication) that an important factor causing cellular resistance to the Rous virus is a latent infection of the cells with a related virus, probably of the avian lymphomatosis group.

It can be shown that these virus variants derive from the standard type as a consequence of a change occurring spontaneously, randomly, and independently of the cells in which the virus is propagated; the variant characteristic is hereditary. These changes are therefore mutations. Their frequency is of the order of 10^{-3} per virus particle, per generation.

That virus mutants can convert identical cells to different morphological types appears to have a considerable significance. On the one hand, in cells maintained *in vitro*, in the absence of virus infection, the morphological type tends to remain fairly constant over the generations, provided external conditions are uniform; on the other hand, it has been shown in one system that cell morphology changes with changes of the genic balance of the cells (*3*). Therefore the morphological type is an expression of the genic setup of the cell. The hereditary modifications of the morphology of the cells caused by the entry of the virus in them are thus likely to reflect a modification of the genic setup of the cell, due to the activity of the viral genome within the cell.

In examining the morphological conversion of the cell, we can now consider another case: that in which a single type of virus is used to infect cells of different types. Conversion still takes place in the various cell types, but the product of it, depending on the cell type, is different. For instance, a virus line isolated by Temin transforms the standard fibroblasts to round cells, the heart fibroblasts to pale triangular cells, and the lung epithelial cells to bricklike cells.

These observations on morphological conversion show, therefore, that the morphological type of a cell infected by the Rous virus can vary considerably, depending on the genotype of the virus and on the original morphological type of the cell before infection. This finding may have some relevance on the problem of the participation of viruses in the origin of cancer in general. In fact, a possible objection to the hypothesis that the cancers are produced by viruses may be found in the considerable variety of morphological cancer types, since one might consider unlikely the existence of a correspondingly large number of different cancer viruses. The results of the studies on the morphological conversion here reported show that this is not true, and that a considerable variety of morphological types can arise when a single virus, with its mutants, infects various types of cells found in an organism.

The described conversion has a practical importance in that on it is based a very efficient method of assay for the virus. This assay method was developed by Temin and Rubin (*4*). It consists in counting the number of foci of transformed cells produced by a virus sample on a monolayer culture of chicken-embryo fibroblasts under suitable conditions. This assay method is as sensitive or more sensitive than methods based on infecting animals; there is, nevertheless, reason to believe that it detects about only 1 to 10 percent of the potentially infective virus particles.

We shall now turn to other consequences of the infection of a cell by the Rous virus. There appear to be changes of physiological type, other than morphological. Several such physiological changes have been reported. One is an increased growth potentiality of the cells, which becomes manifest only under conditions of crowding of the cultures: The converted cells appear to be able to grow further when the normal cells stop growing (Temin, personal communication). Another reported change is an increase in acid production by cultures of converted cells compared to usual cells. Whether this increase is due to an increased aerobic glycolysis of the cells or to greater crowding of the cultures is still uncertain. Also an increase in the ribonucleic acid (RNA) content of the converted cells has been claimed, but not conclusively proved.

Finally, infection by the Rous virus converts the normal cells into virus-carrying and virus-producing cells (*5*). Virus production by the infected cells begins about 14 hours after infection; the rate of virus release increases with time and reaches a maximum 3 or 4 days after infection. Virus release continues through a number of cell generations without preventing the continued growth and division of the cells. This is shown by the fact that cell populations deriving from a single infected cell under conditions preventing extracellular reinfection produce the same amount of virus as an equivalent number of primarily infected cells, and is even more directly demonstrated by the occurrence of division of infected cells kept individually under continuous observation, after virus release has started. It is not known, however, whether all the descendant cells can continue to multiply and release virus indefinitely.

The property of the Rous virus to infect a cell in such a way, that it will release virus at a constant rate and continue to divide normally for many generations, distinguishes it from the majority of the animal viruses; the Rous virus because of this property is considered a moderate virus. It is likely that other viruses, including possibly all tumor-producing viruses, belong to this class; conclusive evidence is still, however, lacking.

From the *in vitro* study of the Rous virus, another relevant property becomes apparent: The virus has a very restricted host range. This property of the virus can be detected also in the animal. The virus originally isolated by Rous was restricted in its action to the connective tissue of a special line of chickens. During subsequent cultivation, variations arose that extended the host range to the connective tissue of other lines of chicken, and later of ducks, and to brain tissue of chickens. *In vitro*, mutants of extended host range were isolated by Temin (personal communication); these mutants are able to grow in genetically resistant chicken cells, and some of them, more rare, are able to grow in duck cells. Thus the *in vitro* experimentation reveals the same general situation as occurs in animals and, in addition, shows that the changes in host range observed in animals are due to the selection of special virus mutants.

I wish now to make some considerations of general and of a somewhat speculative nature on the essential aspects of the process of infection of a normal cell by the Rous virus and of its conversion into a cell with new properties, which we can define as a Rous cell.

A first point that I wish to discuss is the conversion phenomenon. That a virus can convert a cell by infecting it and can impress upon it new properties has been known for some time. Very clear cases of conversion occur in bacteria infected by temperate bacteriophages; a classical one is that of nontoxigenic strains of *Corynebacterium diphtheriae*, which upon infection with a bacteriophage of suitable genetic constitution are converted to toxin producers. In this and other known instances the mere presence of the phage genome inside the host cells is the only requirement for conversion. One may, however, wonder how legitimate it is to compare results obtained in bacteriophage work with findings in the Rous field. Bacteriophages are different from the Rous virus in that their genome is constituted by deoxyribonucleic acid (DNA); furthermore it is established that the genome of temperate phages enters in an intimate relationship with the genome of the host cell; in the Rous virus, on the contrary, there is an RNA virus, for which any intimate interaction with the chromosomal genic apparatus of the cells is not only unproved, but probably unlikely. However, it has been shown that the bacteriophage genome can cause conversion of the host cell even if it does not establish a contact with the host genome (*6*); thus the Rous virus genome can act from some position in the cell not necessarily associated with the chromosomes. Even with this limitation, the genome of the Rous virus can be regraded as integrated with the cell.

One may wonder about the mechanism by which the conversion is carried out. To this effect it is essential to consider that the virus probably brings into the cell a piece of nucleic acid of the length of 5,000 to 10,000 nucleotides. Such a piece of nucleic acid is equivalent in length to a single gene in bacteriophage. It is therefore likely that the virus brings into the cell the information corresponding to one or very few genes; as a consequence, not more than a few new virus-controlled proteins are synthesized in the infected cells. A virus-controlled protein may become incorporated in the surface of the cell, as experimentally found for cells infected with influenza virus (*7*), and then may produce conversion by changing the structure of the cell surface and consequently its shape and function. This idea finds support in phenomena occurring in lysogenic and colicinogenic bacteria, which carry either a phage or phagelike genome; phenomena of this type are the conversion of the surface antigen in *Salmonella*, by a bacteriophage (*6*), and the association of the colicin protein with the surface antigen, also in *Salmonella* (*8*).

The second point of general interest is the nature of the Rous cells, and particularly whether the Rous cells, obtained by converting normal cells by the Rous virus, are truly cancerous. We lack direct conclusive evidence on this point, and it should be appreciated that direct evidence is difficult to obtain. Transplantation experiments would be needed to test the ability of Rous cells produced *in vitro* to grow as cancer cells in the animal; however, the continued release of virus from the Rous cells would complicate these experiments, since, in the animals receiving the transplant, a cancerous growth could be induced by the virus carried with or produced by the transplanted cells.

Some more indirect experimental evidence on the nature of the Rous cells is however available: The development of Rous cell foci *in vitro* occurs in a way that closely resembles, in timing and probability of infection, the development of virus-induced small tumors on the chorio-allantoic membrane of the chicken embryo; Rous cells produced *in vitro* transferred onto an uninfected chorio-allantoic membrane give rise to similar small tumors by their multiplication. Therefore the consequence of a Rous virus infection *in vitro* and in the animal appears to be similar. This conclusion suggests that the Rous cell observable *in vitro* is similar to the first product of virus infection in the animal. This conclusion is strengthened by the similarity in host range of the virus *in vitro* and in the animal, as already discussed.

However, the question whether the Rous cell, defined as the first transformed cell, either *in vitro* or in the animal, is a cancer cell, still remains unanswered. Between the first infection of a cell by the virus and the development of the fatal sarcoma in the animal many cellular divisions take place. As in other virus-induced tumors, the primary growth might be of benign nature and might become transformed into the malignant form at a later stage, by a second event, for instance, a genetic change of some cells.

A considerable amount of work will have to be done before these questions are answered; until then the real nature of the primary Rous cells and their relation to the final sarcoma will be doubtful. However, since, under favorable circumstances, the formation of the sarcoma in the chicken follows promptly the infection with the virus, the primary Rous cells represent at least something very near to the ultimate cancer cell.

In conclusion, I wish to emphasize that the system constituted by the Rous virus and chicken-embryo cells is especially suited for *in vitro* experimental work of oncological nature. This system has two outstanding properties: (1) the ease with which the normal cells can be transformed into Rous cells; (2) the opportunity that it offers, unique in the cancer field, to compare the properties of a normal cell type with those of its direct pathological derivative.

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Problems in Viral-Tumor Therapy[1]

W. RAY BRYAN, *Laboratory of Biology, National Cancer Institute,*[2] *Bethesda, Maryland*

Two of the most important needs for a more adequate animal-testing program to keep pace with the avalanching stockpile of candidate chemical compounds in the field of cancer chemotherapy are: (1) a new order of magnitude in the production of biologically uniform indicator tumors which simulate naturally occurring spontaneous tumors of laboratory animals, and (2) greater sensitivity and precision of the indicator reactions used for detecting antitumor effects. The first of these objectives can already be achieved with several of the established viral tumors of laboratory animals. There is also some preliminary evidence that, under properly controlled test conditions, the viral tumors may provide a basis for significant advances toward the second objective, that is, for increasing both the sensitivity and the precision of antitumor indicator responses.

In a discussion of this same general subject about 2 years ago, Endicott (*1*) pointed out that serious efforts had not been made toward the use of viral tumors for screening or therapeutic testing, though pilot experiments involving the Rous sarcoma and Friend viruses were then under way. The chief reason given for the delay in use of viral tumors was the lack of sufficient knowledge concerning their biology ". . . to permit the chemotherapist to establish a reliable test system."

It will be among the purposes of this discussion to illustrate the types of biological information that are needed with respect to both the virus and the host, in order that the chemotherapist may be able to devise adequate test systems; also, a brief review will be given of results that have been obtained in therapeutic and prophylactic tests involving viral tumors, including advanced information on the pilot studies mentioned by Endicott. The advanced information that will be given was kindly supplied by Doctors Vincent Groupé[3] and Kanematsu Suguira,[4] for the Rous sarcoma and Friend viruses, respectively.

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[1] Presented at the Cancer Symposium, American Chemical Society, Atlantic City, N.J., Sept. 16, 1959.

[2] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

[3] Institute of Microbiology, Rutgers University, New Brunswick, N.J.

[4] Sloan-Kettering Institute for Cancer Research, New York, N.Y.

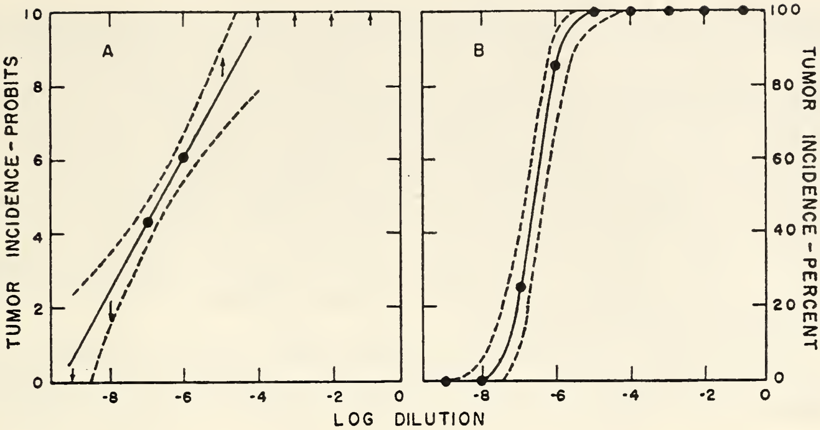
BIOLOGICAL PROBLEMS IN THE PRODUCTION OF TUMORS AND THE DESIGN OF EXPERIMENTS

Tumors induced by viruses simulate naturally occurring spontaneous tumors in that they derive entirely from the hosts' own tissues. They are therefore devoid of divergent histocompatibility antigens which sometimes arise through gene mutations when transplanted tumors are maintained for long periods, even in highly inbred strains of animals, and which may contribute to extraneous antitumor effects that are not observed with indicator tumors of the naturally occurring variety.

Two things are essential to the use of viral tumors in large-scale testing programs: (1) There must be an abundant and economical source of virus of known, or standardized, potency so that there will be no variation in the initiating dose in the production of large experimental lots of indicator tumors. (2) Populations of test animals must be available which are highly susceptible to the virus, and in which the variations among individual animals follow a fairly uniform and predictable distribution about their averages with respect to certain measurable biological properties, such as time elapsing between inoculation of virus and the appearance of a tumor, rate of growth of the tumor, and time-to-death caused by the tumor.

When tumor viruses are first isolated from their natural hosts, some difficulty is usually experienced in obtaining reproducible results on reinoculating the virus into indicator hosts. The reasons for this are varied and are not completely understood, but in several established systems the particular combinations of virus-strain and test-animal population are sufficiently adapted to each other, and the interactions between host and virus in relation to dose are sufficiently stable to permit quantitatively controlled experimentation with chemotherapeutic agents. In the systems in which such stability exists, the data accumulated for demonstrating it can also be used as a basis for planning tests of therapeutic and prophylactic effects.

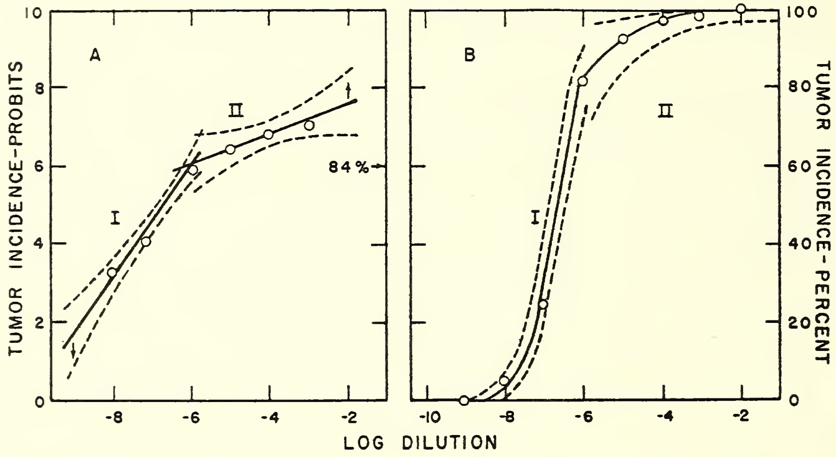
The information needed is the same as that required as background for the development of bioassay methods based upon various biological criteria of response. Thus, in any system it is necessary to know first the dose range within which the incidence of positive responses, *i.e.*, tumors, among groups of inoculated animals decreases from 100 to 0 percent, as well as the dose which on an average just produces, or closely approaches, a 100 percent incidence. It is the relation of this latter dose (in terms of its dilution factor) to the undiluted virus suspension which determines the practicability of a particular virus-host system for the large-scale production of indicator tumors. An example is apparent from a consideration of text-figure 1, the right chart of which illustrates the usual sigmoid percentage-incidence curve obtained with serial dilutions of a virus. The curve represented was obtained with Rous sarcoma virus (*2*). In this experiment the highest dilution giving a 100 percent response was 10^{-5}, but in many experiments carried out over a long period it has



Text-figure 1.—Responses of homogenous groups of chickens to the Rous sarcoma virus—*see* text. (Reproduced from *J. Nat. Cancer Inst.* 16: 856, 1956.)

been observed to vary from about 10^{-6} to about 10^{-4}, due to fluctuations in test-animal susceptibility (*3, 4*). A safe dilution for the routine production of indicator tumors would be 10^{-4}, with a high probability of obtaining tumors in most animals inoculated. Since the log dilution values shown along the abscissa of text-figure 1 are in terms of gram equivalents, the zero value at the right represents the quantity of virus derived from 1 gm. of tumor tissue, suspended in 1 ml. of diluent. The constant volume used for inoculating the chickens was 0.2 ml. for each of the dilutions shown. Thus, if only the 10^{-4} dilution were to be used for chicken inoculations, 1 ml. of the original suspension would provide 10 liters of the desired concentration, or enough for the inoculation of 50,000 test animals. This information is of importance, not because it would ever be desired to inoculate such a large number of animals at any one time, but because the figure represents the total number which eventually could be inoculated with the amount of virus derived from 1 gm. of source tumor tissue, because the virus remains stable upon freezing and storage at temperatures below −50° C. (*3*). It therefore gives a clue to the magnitude of the problem that would be involved in producing adequate quantities of standardized seed virus for use by many laboratories over long periods. In the example cited, each ml. of the original virus suspension could be further divided into 10 subsamples of 0.1 ml. each, for sealing in separate glass ampules, and each ampule would provide enough virus for the inoculation of 5,000 animals; or the original suspension could be diluted 1:10 and 100 subsamples prepared from 1 ml., each being enough for the injection of 500 test animals. Since the processing of 100 gm. of tumor tissue in the preparation of virus suspension is not an undue burden for the average virus laboratory, it is not the preparation of virus itself but the parceling of virus into subsamples that represents the limiting factor in the production of seed virus in most research laboratories. If industrial facilities were available for parceling, identical subsamples of a common virus preparation could be made available in lots of tens of thousands with this particular virus. A given 100 ml. lot of virus suspension would therefore provide enough samples for the eventual inoculation of 5 million chickens for the induction of indicator tumors. The use of such standardized lots is of primary importance in work with the tumor viruses because major fluctuations occur in the yields of virus from different source lots of tumor tissue, and virus suspensions prepared at different times by the same techniques cannot be counted upon to be quantitatively comparable with respect to potency. However, the potency relationships between different lots can be readily established through bioassays.

The left chart of text-figure 1 shows in probits the percentage results given in the right chart. This illustrates the application of a simplified biometric procedure for estimating the dose that would be expected to produce any given percentage response (*2*). Since the approach to 100 percent is asymptotic, the dose estimated to yield a 99 percent response (7.3 probits) may be considered as that which "just produces" a 100 percent response (*5*).



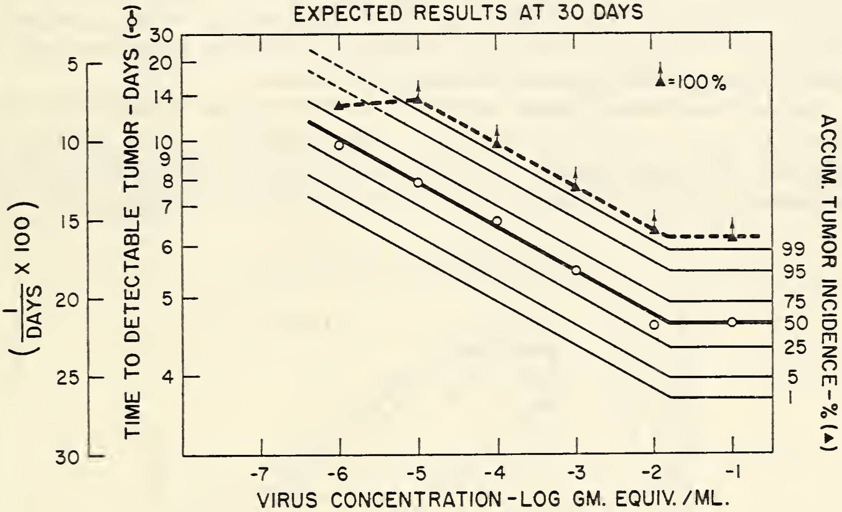
TEXT-FIGURE 2.—Responses of heterogenous groups of chickens to the Rous sarcoma virus—*see* text. (Reproduced from *J. Nat. Cancer Inst.* 16: 859, 1956)

Most strains of chickens are not entirely homogeneous genetically (*see 6–8*), and some lots of test animals will contain individuals of aberrantly high resistance. The dose required to induce tumors in all the individuals, including the highly resistant ones, would therefore be unrealistically high. In this case the percentage results are truncated, as illustrated in text-figure 2, and the dilution to be used for routine production of indicator tumors is estimated by extrapolation of the initial, steep portion of the probit curve to the 99 percent (7.3 probit) level (*see 9*) rather than by taking the dilution actually required to produce a 100 percent incidence. There will, of course, be some loss in experimental animals in this situation since all of them will not develop tumors with the particular dose employed.

A second type of information necessary for production of tumors and the planning of chemotherapeutic tests is the relationship between dose of virus and the time elapsing between virus inoculation and the appearance of tumors, *i.e.*, latent periods. This relationship has two components which must be determined for each dose level: (1) the magnitude of the mean latent period, and (2) the variation of individual latent periods about their mean.

In some experimental systems it is necessary to transform the original time units, *e.g.*, days, to some other function, such as their reciprocals or logarithms, in order to simplify statistical analyses and to facilitate the construction of charts for graphic interpretations (*see 2, 9*). The Rous sarcoma virus in chickens represents such a system.

Text-figure 3 illustrates typical latent period data obtained with successive tenfold dilutions of Rous sarcoma virus. The reciprocal scale along the ordinate at the extreme left was used in the computations and for plotting the data on the chart, but it is the adjacent ordinate scale showing the corresponding points in days that is used for further graphic interpretations. The central solid line represents the computed regression line drawn through the observed means indicated by the open circles. The other diagonal lines were drawn at the respective multiples of the average standard deviation corresponding to the accumulative percentage values shown at the extreme right of the figure. Since the individual latent periods are approximately normally distributed in terms of their reciprocals (*2*), deviates on a normal curve were used as the multipliers.

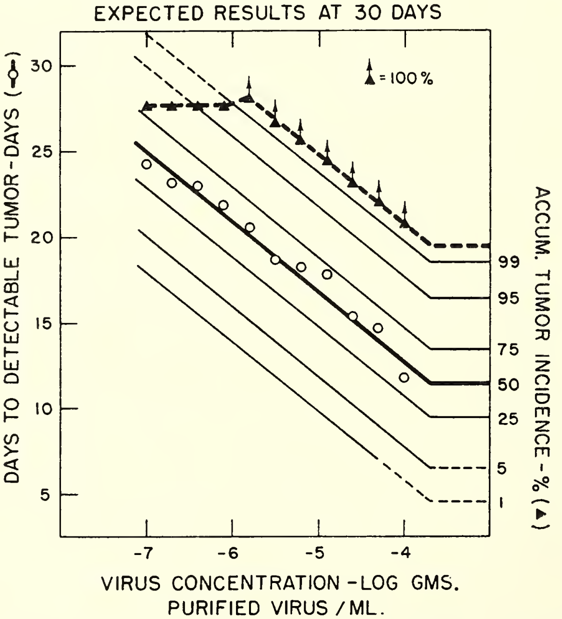


TEXT-FIGURE 3.—Response of homogenous groups of chickens to the Rous sarcoma virus; 0.2 ml. of the dilutions indicated along abscissa inoculated into wing web of 2-week-old New Hampshire Red chickens—*see* text.

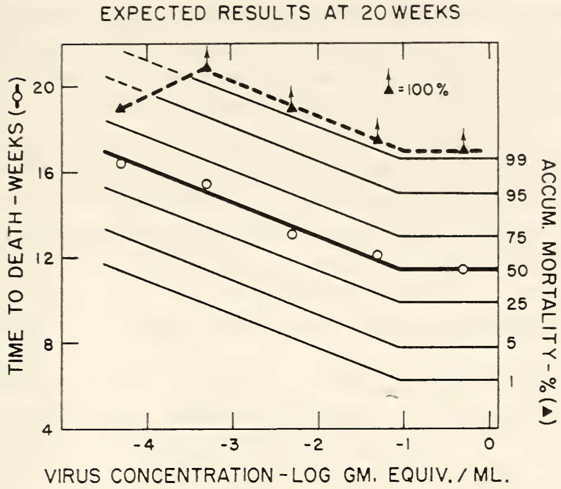
The percentage incidence results are included on the chart for the sake of completion. They are shown as solid triangles plotted in relation to the diagonal accumulative-percentage lines. Since 100 percent values cannot be plotted in terms of normal deviates, they are indicated on the chart by the points with arrows drawn just above the 99 percent line.

Composite charts such as that of text-figure 3 contain most of the information needed for the planning of experiments, including the systematic production of indicator tumors. They take the place of the "preliminary experiment" usually carried out with rapidly acting viruses of other types, for the purpose of quantitative orientation. Such is not possible with the tumor viruses, however, because of the relatively long times required by them to produce their effects, and by the time the preliminary results are obtained, test animals of the same lot are older and may no longer have the same degree of susceptibility to the virus. Since there is some variation in susceptibility among experimental lots of most test-animal populations (*3*), charts of this type cannot be used directly for quantitative analytical interpretations. However, they are entirely adequate for preliminary planning and are usually no more inaccurate than are the results of preliminary titration experiments with other viruses, involving small numbers of animals.

Similar orientation charts are shown in text-figures 4 and 5 for the Shope papilloma virus (*10*) and for a mouse leukemia virus isolated by Moloney (*11*), respectively. In neither of these cases was it necessary to transform the original time units for the purpose of charting, since the



Text-figure 4.—Responses of rabbits to 0.1 ml. inoculums of the Shope papilloma virus—*see* text. Data of (*12*).



TEXT-FIGURE 5.—Responses of BALB/c mice inoculated when 1 or 2 days old with 0.1 ml. volumes of various dilutions of the Moloney leukemia virus—*see* test. Preliminary analysis of data made at the 5th month of an experiment still in progress. Data kindly supplied by Dr. J. B. Moloney of the National Cancer Institute. [*See* (*11*) for final analysis of the results of this experiment.]

original units gave the relationships desired. The dosage scale for the papilloma data (*12*) is in terms of log grams of purified virus per ml., the average yield of purified virus per gm. of wart tissue being about 0.1 mg. (*13*). In contrast to the results of text-figures 3 and 4 for the Rous sarcoma and Shope papilloma viruses, which represent tumor latent periods, the responses of text-figure 5 represent times-to-death with leukemia. Data on the latent period of leukemia development have not yet been published, but it is stated that the average time from palpable spleen and lymph nodes to death with leukemia is about 19 days (*11*).

As may be seen, this leukemia virus can be diluted at least 2,000-fold and will still induce leukemia in 100 percent of the test mice. Since the volume of the diluted virus used for inoculating young mice is only 0.05 to 0.1 ml., the virus derived from 1 gm. of leukemic tissue would be sufficient for the inoculation of 20,000 to 40,000 animals. The production of large numbers of indicator tumors with this virus should therefore be as practical as with the Rous sarcoma virus.

Papillomas produced by the Shope virus have not been used for systematic screening purposes. Possibly the reason for this is that it is a benign wart, and no one is particularly interested in curing benign warts. However, it is not an ordinary wart since it usually persists, rather than undergoing retrogression after a short time as do similar lesions of other species. Most important are the facts that: (1) The virus is of the deoxyribonucleic acid (DNA) type (*14*); (2) it is found primarily in the nuclei of the papilloma cells (*15*), and (3) these benign virus lesions frequently undergo transformation to malignant cancers (*16*). Compounds that would stop the benign lesions might, therefore, have an important bearing on the prevention of cancer, since they would give a clue to the types of substances that are capable of attacking DNA-type viruses in an intranuclear location, and they would remove the precancerous soil, on a basis of which malignancy may later develop.

Since there is evidence that some chemotherapeutic agents are more effective against tumors produced by weaker doses of virus (*17*), it would seem desirable to use for the induction of indicator tumors the weakest doses that will produce tumors in all, or most, of the test animals inoculated. As already pointed out, this is also the dose desired for the conservation of seed virus. When the responses to such doses are measured in weeks, or months, as with the mouse leukemia virus, preliminary information such as that illustrated in text-figure 5 is essential for the efficient scheduling of the animal inoculations, the times for initiating routine examinations for tumors, and the use of animal caging facilities.

Finally, since it is probable that the variations in individual latent periods among animals inoculated with the same dose of virus are associated to some extent with differences in individual test-animal susceptibilities (*6*, *18*), it may be possible to reduce the variations among animals in autonomous chemotherapeutic tests by selecting cohorts of indicator tumors appearing within successive, relatively short intervals of time, the animals being divided within cohorts, at random, into control and test groups for the independent tests. The accumulative frequencies with time, such as those shown in text-figures 3 to 5, would permit the advanced planning of appropriate cohort intervals for a given virus-host system. It has already been established that the tumors which appear earliest in groups of animals inoculated with strong doses of virus yield the largest quantities of recoverable virus on extraction (*6*, *19–21*). Such selected tumors are now commonly used for increasing the potency of virus strains through successive passages (*6*, *11*, *19–22*), and for maintaining high levels of virus in source tumor tissues (*6*, *19–21*). It seems likely that this same relationship may hold also for tumors produced by the weaker doses used in chemotherapeutic tests, and that the precision of antitumor responses may in some instances be increased by the cohort segregation procedure proposed.

METHODS OF CHEMOTHERAPEUTIC TESTING AND PRELIMINARY RESULTS OF PILOT STUDIES

Rous Sarcoma Virus

Attempts to employ virus-induced tumors in therapeutic-type tests date back to the early days of the Rous sarcoma (*23*) and the exploratory studies of Rous (*24*) on the effects of irradiation. However, because of the unpredictability of virus source materials, during earlier years, and the wide variations obtained in the biological results of different experiments (*see 4*), serious consideration was not given, until recently, to the use of this tumor in actual screening and therapeutic-testing programs. During the last few years much has been learned concerning the biology of both the virus and the tumors which it induces, and systematic studies have been begun on the use of virus-induced Rous sarcomas in chemotherapeutic research.

Among the first to investigate this tumor with the newer bioexperimental techniques and standardized preparations of virus were Groupé and his associates (*17*), who studied the tumor-therapeutic and virus-prophylactic effects of the biological product, xerosin (*25*), which had been found to modify the reactions of test animals to certain viruses of infectious diseases (*26*). They found that daily injections of xerosin significantly delayed the appearance of Rous sarcomas in chickens, whether the injections were begun before (prophylactic tests) or after (therapeutic tests) infection with the virus. Tumors induced with strong doses of the virus were not further altered, nor was life prolonged by continued xerosin treatment. On the other hand, tumors induced with weaker doses of the virus were converted to a slowly growing, "atypical" type in more than 50 percent of the test animals. The same result was obtained even when initiation of the treatment of each chicken was delayed until its tumor had become grossly visible. Although a few of the atypical tumors retrogressed, the great majority of them continued to grow slowly and eventually killed their hosts in those experiments in which the animals were maintained for sufficient periods.

The present interest in the studies of Groupé and associates is not so much in xerosin itself as a potential antitumor agent, but in the testing techniques that were developed by these investigators during the study of this and other factors which modify response to the Rous sarcoma virus (*27*, *28*). The latent-period response to this virus had been shown to be a relatively efficient indicator reaction for bioassays, and one that could be subjected to statistical control and interpretation by relatively simple graphic methods (*2*, *9*). Using the information that microscopic tumor foci may be present within 3 days after virus inoculation (*29*), Groupé devised a therapeutic-test procedure in which candidate compounds were administered beginning on the 3d day after virus inoculation. It was anticipated that any antitumor effect on the microscopic foci would be manifested by a delay in the appearance of grossly visible tumors, and that simple daily inspections for the presence of tumors would provide the basis for a highly sensitive and statistically analyzable criterion of antitumor activity. This has proved to be the case. The graphic statistical methods used in analyses of the data were the same as those previously illustrated by Groupé *et al.* (*27*, *28*)—*see* (*9*)—for a biometric discussion.

Table 1 gives examples of types of results, having different degrees of statistical significance, taken from actual preliminary screening experiments carried out in Groupé's laboratory. Although statistically significant delays in the appearance of experimental tumors as compared with controls, such as that illustrated in the bottom row of the table, provide

Table 1.—Types of results in preliminary screening tests based upon latent-period delay of Rous sarcoma (Groupé *et al.*)

Candidate microbial preparation	Number of chicks	Accumulative percent with tumors on day: 4	5	6	7	P	Remarks
(None)	30	3	40	93	97	—	Control
Ex–100	15	7	40	80	93	>0.05	No significant difference
Ex–98	15	0	13	47	87	$0.05>P>0.01$	Borderline significance
Ex–89	15	0	7	53	93	<0.01	Significant

a simple and highly sensitive test for antitumor activity, it should be emphasized that such accumulative percentage values alone do not tell the whole story. The response of a given chicken is scored as positive on the day the tumor appears, and it contributes full weight to the computation of the final accumulative percentage value, regardless of the fact that it may grow very slowly and may be only a small fraction of the size of the control tumors at the termination of the preliminary test.

Similar differences in size, after a period of 10 days, were employed by Johnson and associates (*30, 31*) in another method of measuring antitumor activity with Rous sarcomas induced with standardized doses of the virus. These investigators studied primarily the prophylactic effects of their compounds but were able to demonstrate a high degree of reproducibility of quantitative inhibition, as measured by the weight ratios of experimental to control tumors.

In table 2 are shown the summarized results of prophylactic effects on the Rous sarcoma virus in tests carried out in the laboratories of Johnson and Groupé with compounds that had previously been found to produce antitumor effects in some other, nonviral test system. Although some of the compounds of the separate studies were the same, the series was not identical in the two laboratories. As may be seen, approximately half of those tested in each laboratory were detected by the Rous sarcoma prophylactic tests.

On the other hand, only 3 out of a total of 38 compounds effective in other systems were detected by Groupé and associates in therapeutic tests initiated 3 days after Rous sarcoma virus infection. They are

Table 2.—Results with Rous sarcoma of compounds effective in other systems

Prophylactic (antiviral) effects*

Investigators	Test criterion	Number of compounds giving result: Virus inhibition (+)	No significant inhibition (−)	Total No.
Johnson *et al.*	Tumor weight	11	13	24
Groupé *et al.*	Latent-period delay	11	10	21

*Daily injections begun 1 or 2 days before virus infection.

listed in table 3. It is of interest that the 3 compounds shown are among those which have been found to be most effective in the actual treatment of neoplasms in human subjects.

TABLE 3.—Results with Rous sarcoma of compounds effective in other systems (Groupé *et al.*)

Therapeutic (antitumor) effects*	
Chemical compound	Latent-period delay test
Amethopterin	$P<0.01$
6-Mercaptopurine	$P<0.01$
Triethylenemelamine (TEM)	$.05>P>0.01$
35 other compounds	No significant delay

*Daily injections begun 3 days after virus infection.

In a pilot screening program in search of effective microbial products in cultures of Actinomycetes and gram-negative bacteria, 8 culture fractions (supernatants, filtrates, or cell extracts) were found to produce latent-period prolongations of borderline significance ($0.05>P>0.01$), and 6 were found to produce highly significant prolongations ($P<0.01$) out of a total of 245 examined by the preliminary therapeutic test. More critical studies on these 6 most effective products are now under way.

Friend Leukemia Virus

Detailed data that would permit the construction of orientation charts, *e.g.*, text-figures 3 to 5, for the Friend virus (*32*) have not been published, but it is among the most rapidly acting tumor viruses and is comparable to those of Rous sarcoma and Shope papilloma. Pilot screening studies involving the Friend leukemia have been carried out by Suguira, and are to be reported by him in a paper now in press (*Gann*, September, 1959). The following information, including the summary of results contained in table 4, was kindly supplied by Dr. Suguira.[5]

TABLE 4.—Eight compounds among over 200 tested which showed the ability to inhibit Friend virus leukemia (Suguira)

1. Mitomycin c
2. Triethylenemelamine (TEM)
3. Thioguanine
4. Methanesulfonic acid, tetramethylene ester (Myleran)
5. Estradiol
6. Methoxyn
7. Methanesulfonic acid, nonamethylene ester [1,9-Di(methanesulfonoxy)nonane]
8. 9α-Fluoro-2-α-methylhydrocortisone acetate

The 8 compounds listed in table 4 were those found to be effective among over 200 compounds examined in preliminary therapeutic tests in which the administration of candidate compounds was initiated 24 hours after the inoculation of mice with Friend leukemia virus. The criterion of antitumor activity was the ratio of the weights of spleens of the treated animals to those of controls, 2 weeks after the inoculation of virus. Four of the compounds that were found to be the most effective in this preliminary test (#1, 2, 4, and 7 of table 4) were subjected to follow-up tests in which the administration of the compounds was delayed until 5 days after virus inoculation. Neoplastic cells can be detected by microscopic examination of the spleens of control inoculated mice at this time. All 4 compounds were also effective by the more severe therapeutic test. In some cases the spleens of treated mice weighed less than 0.2 gm. and could not be differentiated from the normal spleens of uninoculated mice, whereas the spleens of the inoculated controls were greatly enlarged and weighed from 2 to 4 gm. at the termination of the test. Again, the compounds that were effective in this viral-tumor screening system were among those that have been found to be most effective in other systems.

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[5] *See* footnote 4.

Other Tumor Virus-Host Systems

The detailed biological information necessary for the planning and carrying out of chemotherapeutic research is also available for 3 additional tumor virus-host systems: the virus-induced fowl leukoses, myeloblastosis (*33*), erythroblastosis (*34*), and lymphomatosis (*35*). However, systematic studies have not yet been made on the potential value of these systems as chemotherapeutic tools.

CONCLUDING COMMENTS

Sufficient knowledge of the biological properties of several well-established tumor virus-host systems has now been achieved to permit them to be used in systematic chemotherapeutic research. Pilot studies by Groupé and Johnson and their associates on the Rous sarcoma virus, and by Suguira on the Friend leukemia virus, indicate that highly efficient, reproducible, prophylactic, and therapeutic tests can be carried out with both of these systems under practical conditions. The screening methods in both systems were capable of distinguishing several of the chemotherapeutic compounds which previously had been found to be among the most effective in other nonviral tumor test systems.

These findings should lend encouragement to a full-scale test program involving viral tumors, in the further search for more effective antitumor agents.

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